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Nutrition of Animal Cells in Tissue Culture. I. Initial Studies on a Synthetic Medium.*† (17557)

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Over the past 30 years, the nutritive media that have been employed for the propagation of animal cells in tissue culture have been derived, directly or indirectly, from the organism and have consisted of blood plasma, blood serum, body exudates and extracts of various tissues and organs. But the complexity and variability of these natural substrates has made it almost impossible to employ them in experiments designed to determine the substances that are actually utilized by the cells for survival and multiplication. From time to time, attempts have been made to devise nutritive media comprised of chemically-known ingredients. Thus, Vogelaar and Erlichman(1), Baker(2), and Baker and Ebeling(3), have reported feeding solutions for fibroblasts, epithelial cells and blood macrophages. These media consisted in part of chemically-defined substances, but the addition of serum or other natural substrates was necessary for cell multiplication and survival. Fischer and his associates(4) employed dialyzed plasma and dialyzed chick embryo extract as a base and devised supplementary solutions consisting of compounds of known biological importance to replace the accessory growth factors removed during dialysis. White(5) reported a culture fluid consisting of constituents of known composition that supported the life of relatively large masses of chick embryo tissue for several weeks. It was found by Jacoby and Darke(6), however, that it was necessary to add serum to White's mixture in order to support the life and activity of pure cultures of blood macrophages.

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* This investigation was supported, in part, by grants from the National Cancer Institute of Canada, and the Ontario Cancer Treatment and Research Foundation.

† Grateful acknowledgment is made to Mrs. M. G. Pearce and Mrs. C. J. Porter for technical assistance.

1. Vogelaar, J. P. M., and Erlichman, E., *Am. J. Cancer,* 1933, v18, 28.

2. Baker, L. E., *Science,* 1936, v83, 605.

3. Baker, L. E., and Ebeling, A. H., *J. Exp. Med.,* 1939, v69, 365.

4. Fischer, A., Astrup, T., Ehrensvard, G., and Oehlenschlager, V., PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 40.

It is the purpose of the present communication to report the first of a series of studies made in this laboratory over the past 2 years in an attempt to devise a synthetic medium of chemically-known composition that would promote continuous cell multiplication of small amounts of tissue in the absence of blood serum and embryo extract. Although a completely adequate synthetic mixture has not yet been achieved, the results thus far obtained are sufficiently encouraging to justify the present report.

Materials and Methods. All cultures were prepared from the leg muscle of 11-day-old chick embryos. After the tissue had been chopped with cataract knives into extremely fine fragments, and the fragments had been washed with Earle's modification of Tyrode's solution(7), very small amounts of the resulting suspension were transferred with a pipette to the inner surface of standard pyrex test tubes (18 × 150 mm). The culture fluid (2 ml) was then placed in the bottom of the tubes, care being taken to avoid wetting the tissue, the pH was adjusted to 7.2 with a gas mixture consisting of 8% CO_2, 21% O_2 and 71% N_2, and the tubes were tightly stoppered. It was found that a period of 1 hour was required for the tissue to become firmly attached to the glass surface of the tubes before the fluid medium could be washed over it. As soon as the tissues were flooded with the culture fluid, the tubes were inserted in an almost horizontal position in a rotating drum(8) at 38°C.

All cultures received a preliminary feeding mixture containing naturally-occurring ingredients (horse serum and embryo extract) when first made and were left in the rotating drum for an initial period of 3 to 5 days. At the end of this time, they were examined microscopically and any unsuitable cultures were discarded. The remaining cultures were divided into groups of 5 or more, and the preliminary feeding mixture was replaced by various synthetic mixtures. These mixtures were changed 3 times a week. The cultures were examined microscopically at frequent intervals and careful record was made of the development of new tissue, breakdown of the original tissue, and the general appearance of the individual cells. Cultures were maintained in the synthetic mixtures until living cells were no longer apparent. The average survival time of the cultures in each group of a series (Table I) was considered to be a rough indication of the suitability of the experimental media. Throughout the experiments, certain cultures were continued in the preliminary feeding mixture (containing horse serum and embryo extract) as positive controls, and in Earle's solution alone as negative controls.

By careful refinements of technique, it was found possible to decrease progressively the amount of embryo tissue added to the roller tubes. In all of the experiments to be reported, cultures were made with the minimum amount of tissue (Fig. 1) that would yield extensive cell growth.

The non-synthetic, *preliminary feeding mixture* consisted of 4 parts horse serum, 6 parts

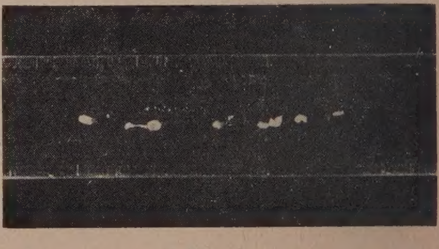

FIG. 1.

Portion of 18 × 150 mm test tube showing amount of tissue employed in preparation of typical culture; photographed after 18 hr incubation. ×1.

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5. White, P. R., *Growth*, 1946, v10, 231.

6. Jacoby, F., and Darke, S. J., *Nature*, 1948, v161, 768.

7. Earle, W. R., *J. Nat. Cancer Inst.*, 1943, v4, 165.

8. Gey, G. O., *Am. J. Cancer*, 1933, v17, 752.

Earle's solution, and final concentrations of 1% chick embryo extract(9) (in Earle's solution) and 0.002% phenol red.

The *components of the synthetic media* were of the highest grade obtainable commercially and were employed without further purification. It was found most convenient to prepare a number of basic stock solutions and to combine these in various proportions.

Sol. A: *l*-Arginine monohydrochloride, 14.0 mg; *l*-histidine monohydrochloride, 4.0 mg; *l*-lysine monohydrochloride, 14.0 mg; *dl*-tryptophane, 4.0 mg; *dl*-phenylalanine, 10.0 mg; *dl*-methionine, 6.0 mg; *dl*-serine, 10.0 mg; *dl*-threonine, 12.0 mg; *dl*-leucine, 24.0 mg; *dl*-isoleucine, 8.0 mg; *dl*-valine, 10.0 mg; *dl*-glutamic acid monohydrate, 30.0 mg; *dl*-aspartic acid, 12.0 mg; *dl*-α-alanine, 10.0 mg; *l*-proline, 8.0 mg; *l*-hydroxyproline, 2.0 mg; glycine, 10.0 mg; *l*-glutamine, 20.0 mg; sodium acetate, 16.3 mg. These ingredients were dissolved in 100 ml of double-strength Earle's solution.

Sol. B: *l*-Tyrosine, 200 mg; *l*-cystine, 100 mg. These substances were dissolved with gentle heating in 100 ml of 0.075 N HCl.

Sol. C: Niacin, 25.0 mg; niacin amide, 25.0 mg; pyridoxine hydrochloride, 25.0 mg; pyridoxal hydrochloride, 25.0 mg; thiamin hydrochloride, 10.0 mg; riboflavin, 10.0 mg; calcium pantothenate, 10.0 mg; *i*-inositol, 50.0 mg; *p*-aminobenzoic acid, 50 mg; choline chloride, 500.0 mg; glass-distilled water to 200 ml.

Sol. D: Cysteine hydrochloride, 100.0 mg; glutathione, 50.0 mg; ascorbic acid, 50.0 mg; glass-distilled water to 100 ml. A fresh solution was prepared each month.

Sol. E: *d*-Biotin, 10.0 mg; glass-distilled water to 100 ml.

Sol. F: Folic acid, 10.0 mg; Earle's solution to 100 ml.

Sol. G: Vitamin A, 10.0 mg; ethyl alcohol (95%), 1.0 ml; Tween 80 (5% aqueous solution), 10.0 ml; glass-distilled water to 100 ml. A fresh stock solution was prepared each month.

Sol. H: Calciferol (vit. D_2), 10.0 mg; ethyl alcohol (95%), 1.0 ml; Tween 80 (5% aqueous solution), 10.0 ml; glass-distilled water to 100 ml.

Sol. I: Disodium α-tocopherol phosphate,‡ 10.0 mg; glass-distilled water to 100 ml.

Sol. J: Menadione (vit. K), 10.0 mg; glass-distilled water to 100 ml.

Sol. K: Cholesterol, 10.0 mg; ethyl alcohol (95%), 10.0 ml; Tween 80 (5% aqueous solution), 10.0 ml; glass-distilled water to 100 ml.

Sol. L: Adenine sulfate, 500.0 mg; glass-distilled water to 100 ml.

Sol. M: Guanine hydrochloride, 10.0 mg; xanthine, 10.0 mg; hypoxanthine, 10.0 mg; thymine, 10.0 mg; uracil, 10.0 mg. These substances were dissolved in 100 ml glass-distilled water with the addition of 0.35 ml concentrated NH_4OH.

Sol. N: *d*-Ribose, 100.0 mg; *d*-2-desoxyribose, 100.0 mg; glass-distilled water to 100 ml. A 1.0 ml amount of this solution was incorporated in each liter of Solution A.

Sol. O: Adenylic acid (from muscle), 100.0 mg; glass-distilled water to 100 ml. A 4.0 ml portion of this solution was incorporated in each liter of Solution A.

Sol. P: Adenosinetriphosphate (dibarium salt), 67.6 mg. This substance was dissolved in 50 ml of 0.01N HCl. The barium was precipitated by the addition of a slight excess of saturated Na_2SO_4 solution, removed by centrifuging, and the clear supernatant was diluted to 100 ml with glass-distilled water. A fresh solution was prepared each week.

Sol. Q: $Fe(NO_3)_3 \cdot 9H_2O$, 14.5 mg; glass-distilled water to 100 ml.

Stock solutions A, B, K, L, M, P and Q were employed without further dilution. Solutions C, G, H and J were diluted 1:10 in glass-distilled water before use. Solutions D, E, F and I were diluted 1:100 in glass-distilled water.

All solutions used in the preparation of the synthetic mixtures were sterilized by passage through UF fritted glass filters (Corning). Stock solutions and the combined mixtures were stored in low-actinic glassware. The

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9. Morgan, J. F., and Parker, R. C., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 665.

‡ Obtained through the courtesy of Dr. J. G. Baxter, Distillation Products, Inc., Rochester, N.Y.

TABLE I.
Average Survival Time of Chick Embryo Roller-tube Cultures in 6 of the 199 Synthetic Mixtures That Were Tested.

Mixture No.	Ingredients	No. of experiments	Total No. of cultures*	Avg survival (days)
Balanced salt mixture	Earle's modification(7) of Tyrode's solution (incorporated in all subsequent mixtures)	6	23	6.6
22	Amino-acid mixture	5	23	14.5
81	Mixture No. 22 + vitamins and Tween 80	5	18	16.8
88	Mixture No. 81 + purines, pyrimidines and cholesterol	5	22	23.4
148B	Mixture No. 88 + ATP, adenylic acid, sodium acetate, glutamine, ribose, and desoxyribose	12	70	28.0
199	Mixture No. 148B + ferric nitrate	11	60	33.0†

* Approximately 3,000 cultures were used to test all of the mixtures studied.
† A few cultures in this mixture have survived beyond 70 days.

majority of these solutions were kept at 4°C, but certain stock solutions, *e.g.* adenine, cholesterol, tyrosine and cystine, which tended to form precipitates in the refrigerator, were stored at room temperature.

In the preparation of the synthetic mixtures, 25 ml of the amino acid basal mixture (Solution A) were taken, and to this were added appropriate volumes of the other stock solutions. Sufficient glass-distilled water was then added to bring the total volume to 50 ml. The isotonicity of the media was confirmed by freezing-point determinations. Each new ingredient, or group of ingredients, added to the basal solution was first studied over a wide range of concentrations to detect possible toxic effects, and was finally incorporated in the mixture at a level that was either beneficial or, at least, non-toxic.

Experimental. Amino acids. Preliminary experiments were carried out in which an acid hydrolyzate of casein, fortified with tryptophane, served as the sole source of nutrient. This solution proved to be completely inadequate, since it supported cell life for only 5 to 7 days. An amino acid mixture was then prepared from pure constituents and was made up in proportions based on analyses of tissue proteins(10). This mixture was tested at several concentrations over the range of 500 to 50 mg/100 ml total amino acids. A level of 100 mg/100 ml was found to be most suitable. At this concentration, cultures remained alive for an average period of 14 days (Table I), while at levels above and below this amount the duration of life was somewhat shorter.

Vitamins. The basal amino acid mixture was next supplemented by the addition of vitamins (Solutions C to J). These were tested over a wide range of concentrations, some singly and some in groups. The addition of vitamins was found to improve the appearance of the individual cells, but was not observed to increase the amount of new tissue.

In these early vitamin experiments, considerable difficulty was encountered with vitamins A and D. The stock solutions of these substances were made up in 95% ethyl alcohol, and the vitamins tended to precipitate out when they were added to the aqueous basal mixture. It was also observed that the presence of more than a trace of these solutions

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10. Block, R. J., and Bolling, D., *The Amino Acid Composition of Proteins and Foods,* Springfield, Ill., Thomas, 1945.

markedly decreased the survival period of the cultures. This was attributed to the toxic effect of ethyl alcohol upon the tissue cells.

Tweens. The surface active agents of the Tween series were next investigated as a possible means of supplying the tissues with a water-soluble source of fatty acid. Extensive experiments, to be published separately, have established that concentrations ranging from 0.005 to 0.0005% were suitable for use in the cultures. Since it was considered possible that the cells might require a source of *unsaturated* fatty acid, it was decided to incorporate Tween 80 (containing oleic acid) in all subsequent synthetic mixtures. The surface activity of this compound was also utilized to dissolve fat-soluble compounds. It was found that if vitamin A, vitamin D and cholesterol were first dissolved in a minimal amount of 95% ethyl alcohol, and Tween 80 was added subsequently (Solutions G, H, K), these substances remained in solution and did not precipitate even in high dilution with distilled water.

The addition of vitamins A and D, in Tween 80, to the synthetic mixture was found to prolong the survival time of the cultures and to improve the appearance of the cells. Whether this beneficial effect can be attributed to the Tween 80 or to the elimination of all but minute traces of alcohol from the media has not yet been established.

Nucleic Acid Constituents. Since the synthetic mixture now contained amino acids, vitamins and a source of fatty acid, attention was next directed to the constituents of nucleic acid, with particular emphasis upon the purine and pyrimidine bases. Adenine sulfate was not found to exert any marked effect when tested alone at concentrations from 10 to 0.1 mg/100 ml. A purine and pyrimidine solution, containing guanine hydrochloride, xanthine, hypoxanthine, thymine and uracil, was found to be toxic at 6.0 mg/100 ml, but to be slightly beneficial at 0.6 and 0.06 mg/100 ml. Adenine sulfate at the 1.0 mg/100 ml level, however, resulted in some improvement when added to a mixture in which the purines and pyrimidines had already been incorporated. The pentose sugars, ribose and desoxyribose, did not appear to influence the survival time of the cultures, but were added to the medium in order to complete the list of nucleic acid components.

Accessory Growth Factors. In addition to the several groups of compounds already mentioned, a number of accessory growth factors were also tested for possible stimulating effects upon the cultures; and these were incorporated in the synthetic mixture. Even though some of them showed no apparent beneficial effect when tested singly, there was a progressive improvement that could be attributed to various combinations of these metabolites.

Cholesterol was dissolved in Tween 80, as described previously, and was added to the basal mixture at concentrations from 2.0 to 0.02 mg/100 ml. No effect upon the cultures was noted.

Sodium acetate was tested at concentrations ranging from 5.0 to 0.0005%. Levels of 5.0 and 0.5% were found to be toxic, while lower concentrations had no appreciable effect.

An adenosinetriphosphate (ATP) solution was prepared, as already described, and this compound was tested on the cultures at levels of 100 to 0.01 μg per ml. No effect upon the cultures was noted at any concentration studied.

Adenylic acid (from muscle) was used over the same range of concentrations employed with ATP, but it had no apparent effect upon the cultures. A number of experiments were carried out in which the concentrations of ATP and of adenylic acid were varied in respect to each other. Under these conditions, also, no appreciable effect upon the cultures was observed.

Glutamine was incorporated in the synthetic mixtures at concentrations ranging from 100 to 10 mg/100 ml, and appeared to be slightly beneficial at the highest level tested. Later experiments indicated that this concentration was not effective when added to synthetic mixtures containing ATP and adenylic acid. In the presence of these metabolites, however, considerable improvement was exerted by a lower level of glutamine. This beneficial effect was greater than that obtained previously by the highest level of glutamine in the absence of ATP and adenylic acid. Glutamine, ATP and adenylic acid were finally added to the synthetic mixture in the proportions that had given this marked improvement.

Trace Metals. Although it is planned to study the possible effects of adding various trace metals to the synthetic mixture, iron is the only one that has so far been tested. Solutions of ferric nitrate and ferrous sulfate were tested over wide ranges of concentration. The results appeared to indicate that low concentrations of both salts were slightly beneficial. Ferric nitrate was finally selected for use in the synthetic mixture (Table I) because it appeared to be the more stable of the two.

Synthetic Mixture No. 199. Since these experiments had established the optimal concentrations of the individual substances tested, it was now possible to combine them as shown in Table II. Embryo tissue cultivated in this mixture was found to put out extensive areas of new growth. This was in striking contrast to the results obtained with simpler mixtures. The majority of the cells that grew out from the original tissue fragments cultivated in Mixture 199 were large, flat and spindle-shaped. They did not develop excessive granulation for about 3 weeks, after which time many of the cultures underwent progressive degeneration.

In repeated experiments, Mixture 199 supported cell life for an average period of 4 to 5 weeks, and certain cultures survived for more than twice that time. Further studies are being undertaken in order to determine, if possible, the additional substances that may be required to make Mixture 199 completely adequate for continuous cell multiplication. The effect of supplementing this medium with small amounts of serum and embryo extract is also under investigation.

Discussion. The studies reported in the present communication, which include the results obtained with approximately 3000 cultures, have shown a progressive improvement in the survival of the tissues that have been cultivated. Although the medium that has been developed is not entirely adequate, it will support cell life for an average period of 4 to 5 weeks and has made it possible to make a number of interesting observations on cell nutrition.

TABLE II.
Synthetic Mixture No. 199.*

	Mg per 1000 ml
l-Arginine	70.0
l-Histidine	20.0
l-Lysine	70.0
l-Tyrosine	40.0
dl-Tryptophane	20.0
dl-Phenylalanine	50.0
l-Cystine	20.0
dl-Methionine	30.0
dl-Serine	50.0
dl-Threonine	60.0
dl-Leucine	120.0
dl-Isoleucine	40.0
dl-Valine	50.0
dl-Glutamic acid	150.0
dl-Aspartic acid	60.0
dl-Alanine	50.0
l-Proline	40.0
l-Hydroxyproline	10.0
Glycine	50.0
Cysteine	0.1
Adenine	10.0
Guanine	0.3
Xanthine	0.3
Hypoxanthine	0.3
Thymine	0.3
Uracil	0.3
Thiamin	0.010
Riboflavin	0.010
Pyridoxine	0.025
Pyridoxal	0.025
Niacin	0.025
Niacinamide	0.025
Pantothenate	0.01
Biotin	0.01
Folic acid	0.01
Choline	0.50
Inositol	0.05
p-Aminobenzoic acid	0.05
Vitamin A	0.10
Calciferol (Vit. D)	0.10
Menadione (Vit. K)	0.01
a-Tocopherol phosphate (Vit. E)	0.01
Ascorbic acid	0.05
Glutathione	0.05
Tween 80 (oleic acid)	20.0
Cholesterol	0.2
Sodium acetate	50.0
l-Glutamine	100.0
Adenosinetriphosphate	10.0
Adenylic acid	0.2
Iron (as ferric nitrate)	0.1
Ribose	0.5
Desoxyribose	0.5

* This mixture also contained a modified Tyrode's solution.

The amino acid requirements of tissue cells have been investigated extensively by Fischer(11), who has shown that cystine is most essential and that arginine, tryptophane, glu-

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11. Fischer, A., *Biochem. J.*, 1948, v43, 491.

tamic acid and lysine are also required. On the basis of Fischer's work, the present finding that a mixture of 19 amino acids is greatly superior to an acid hydrolyzate of casein might possibly be attributed to an increased level of cystine, since casein is known to be deficient in this particular amino acid(10). Moreover, Fischer's results indicate that the concept of "essential" and "non-essential" amino acids does not apply to tissue cultures. Hence, the inclusion of all the naturally-occurring amino acids would seem to be a desirable feature of Mixture 199. It should also be pointed out that the medium reported by White(5) contains only the "essential" amino acids and does not include cystine.

The amino acid mixture finally employed in the present experiments contained 11 *dl* (the more easily available) amino acids and 7 of the natural *l* configuration. Comparative tests were carried out with a similar mixture made up entirely of *l* amino acids.§ But since experiments made with the same range of concentrations failed to show any significant differences in the results obtained with the two solutions, it was concluded that the *d* amino acids were not growth inhibiting.

The present studies did not afford any clear-cut evidence that vitamins are required by cells in tissue culture. It seemed advisable, however, to include a complete vitamin supplement in Mixture 199 in the hope that beneficial effects might be evident when the mixture became more complete. It was felt that the preliminary media should include as many known nutritional factors as possible, even though many of them had no apparent effect. If an adequate synthetic medium could be devised in this manner, the unnecessary substances could eventually be eliminated. The results shown in Table I would seem to justify this method of approach.

The addition of nucleic acid constituents, the purines, pyrimidines and pentose sugars, to the basal solution was found to be beneficial. The incorporation of these compounds into Mixture 199 would seem to constitute an improvement over other synthetic mixtures that have been devised. White's medium contained no nucleic acid derivatives; and Fischer's solution included only hypoxanthine and inosinic acid. The important role that has been assigned to the nucleic acids in cellular metabolism(12,13) would suggest that any synthetic medium for animal tissue cells should contain either the nucleic acids themselves or substances from which they may be synthesized by the cells.

It was hoped that the addition of various intermediary metabolites and accessory growth factors to the basal synthetic mixture might increase very greatly the survival time of the cultures through a stimulation of the cellular enzyme systems involved in the synthesis of protoplasm. For this reason, great importance was attached to the incorporation of adenosinetriphosphate, which is known to activate many metabolic processes(14). The fact that this compound, and also adenylic acid, failed to exert significant effects upon the survival time of the cultures was rather disappointing. It is possible, however, that stimulation by ATP may occur only in the presence of phosphorylated intermediates of the glycolytic cycle. Thus, Fischer and his associates(4) found that whereas ATP did not stimulate growth, fructose diphosphate was definitely beneficial. A further study of this interrelationship is being conducted at the present time.

The addition of glutamine to the basal mixture was observed to increase the life span of the cultures, but the improvement observed was not as great as that reported by Fischer and his associates(4). It should be pointed out, however, that the present experiments were made with a basal mixture that contained a high level of glutamic acid, whereas Fischer's results were obtained in the absence of glutamic acid.

When the present studies were undertaken, it was anticipated that certain interrelationships and possible antagonisms might develop between various groups of growth factors. The only instance of such effects encountered up to the present time is that between glutamine and ATP plus adenylic acid. No explanation of this interesting relationship is available at the present time.

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§ *l*-Threonine and *l*-valine were obtained through the courtesy of Dr. Marvin Darrach, Merck and Co., Ltd., Montreal, Canada.

12. Davidson, J. N., *Symposia Soc. Exp. Biol.*, 1947, v1, 77.

13. Davidson, J. N., *Cold Spring Harbor Symposia Quant. Biol.*, 1947, v12, 50.

14. Potter, V. R., *Advances in Enzymology*, 1944, v4, 201.

The necessity of cultivating the tissues in naturally-occurring nutritive media for 3 to 5 days prior to the replacement of these natural media by synthetic mixtures is an undesirable feature of the present experiments. During this preliminary period of cultivation, the cells might be able to store up reserve food substances that would serve as a source of nutrient during the subsequent period of cultivation in synthetic media. Still, tissues cultivated in Earle's solution alone degenerated and died within 6 or 7 days (Table I). This would seem to indicate that the effects of the initial period of maintenance in the preliminary feeding mixture are not exerted long after this original fluid is removed. Eventually it is hoped that this early maintenance period in non-synthetic media may be eliminated.

The systematic addition of new ingredients to the synthetic mixture has seldom resulted in a marked improvement that could be attributed to any one substance. But periodic comparisons of the effect of the more recent test solutions with simpler mixtures that had been used earlier (Table I) have shown a progressive increase in the survival time of the cultures and a marked improvement in their appearance. Thus, for example, results obtained with Mixture 199 were vastly superior to those obtained with mixtures containing only amino acids, vitamins and Tween 80.

The results reported in the present communication have established that synthetic Mixture 199 will support the survival of chick embryo tissues for an average period of 4 to 5 weeks. Although this mixture will not support the cells indefinitely, it does appear suitable for use as a basal synthetic medium for further investigations on cell nutrition.

Summary. The nutrition of animal cells in tissue culture has been studied with particular reference to amino acids, vitamins, nucleic acid constituents and various accessory growth factors. The optimal concentrations of these substances for the maintenance of cell life *in vitro* have been established and a completely synthetic feeding solution has been devised. Although this mixture will not support cell life indefinitely, it has been found adequate to maintain chick embryo cells for an average period of 4 to 5 weeks. The mixture is being used as a basal synthetic medium for further studies on the unidentified growth-promoting substances known to occur in natural media.

Received October 4, 1949. P.S.E.B.M., 1950, v73.

III. Influence of Renal Tissue in Inhibition of Protein Catabolism.* (17558)

EDWARD C. PERSIKE (Introduced by Arthur L. Bloomfield)

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Data are presented elsewhere(1) which show that the rate of protein catabolism, as measured by the rate of urea formation, is increased markedly in rats after sudden loss of 75% or all of the total renal tissue. A higher rate of protein catabolism was found in rats subjected to complete nephrectomy than in those with 25% of the total renal substance remaining. It was suggested that loss of 75% or all of the total renal tissue might release the mechanism responsible for

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* This work was supported by a grant from the Columbia Foundation.

The author gratefully acknowledges technical assistance by Miss H. J. Ureen, Mr. W. Lew, Mr. L. J. Poo, and Mr. W. Wong.

1. Persike, E. C., and Addis, T., *Am. J. Physiol.*, 1949, v158, 149.

breakdown of body protein from one of its usual inhibitory influences. The experiments reported here support this hypothesis.

Methods. The experimental conditions and methods, and data obtained from control animals are given in detail elsewhere.(1) One hundred and fifty female albino rats, selected at body weights of 150 g, were divided into groups of 10 rats each. The rats of 9 groups were submitted to total nephrectomy. Bilateral ureteral ligations, with division of the ureters between ligatures, were performed on the rats of 6 groups. The rats were fed a calorically adequate solution of 15% glucose in water with 0.4% sodium chloride and vitamins of the B complex for 2 days before operation, and after operation until autopsy, in order to eliminate the influence of food protein consumption on the serum urea concentrations. Three groups of nephrectomized animals were killed at the end of the 4th, 16th and 24th postoperative hours, and 3 groups of rats subjected to bilateral ureteral ligation were killed at the end of the 12th and 24th postoperative hours. The animals were killed by exsanguination under ether anesthesia, and aliquot portions of serum were combined to form one serum pool for each group. Serum urea concentrations were determined by the urease aeration method of Addis,(2) all measurements being made in duplicate. Rates of urea formation were calculated by the procedure used in other experiments.(1)

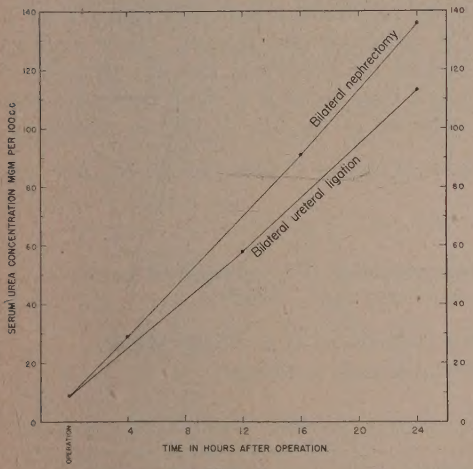

Fig. 1.
Average serum urea concentrations.

Results. Fig. 1 shows that the serum urea concentrations both of the nephrectomized animals and of the rats subjected to ureteral ligation continuously increased after operation until the end of the 24th postoperative hour. The serum urea concentration of the nephrectomized rats, however, increased at a more rapid rate. At the end of the 24th postoperative hour it exceeded the serum urea concentration of the rats whose ureters were ligated by 22.7 mg per 100 cc, or approximately 20%.†

The rates of urea formation are given by Table I. In each successive postoperative 4 hour period, the rate of urea formation of the nephrectomized rats was greater than that of the animals subjected to bilateral ureteral ligation. The total amount of urea formed during the 24 postoperative hours was 120 mg per rat by the nephrectomized animals, and 99 mg per rat by the rats with ligated ureters. The magnitude of this difference cannot be explained by supposing inequalities in hydration, because both types of rats gained an equal amount of weight after operation, and presented grossly equal quantities of subcutaneous and intraperitoneal fluid at autopsy.

Discussion. While the excretory function of the kidneys ceases after bilateral ureteral ligation, blood continues to flow through the organs, and the renal tissue remains viable for

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2. Addis, T., *J. Lab. and Clin. Med.*, 1925, v10, 402.

† The difference between the observed means at the end of the 24th postoperative hour, or 22.7 mg per 100 cc of serum, was tested by Fisher's *t* test. The ratio of the difference to its sampling error was $22.7/9.64 = 2.35$. Entering Fisher's table with 4 degrees of freedom, and halving the probability found, one finds a probability of approximately 4 in 100 that the difference obtained in the plus direction would occur by chance if the experiments were repeated. Furthermore, the observations made at the end of the 12th hour after ureteral ligation also fall to about the same degree below the nephrectomized level. For these reasons, the difference observed between the serum urea concentrations of the nephrectomized rats and of the animals subjected to bilateral ureteral ligation is considered to be statistically significant.

TABLE I.
Rates of Urea Formation.
(Mg per rat per 4 hr period, corrected to 150 g body weight).

	Rates of urea formation		
Hr after operation	Mock operated controls	Complete nephrectomy	Bilateral ureteral ligation
0– 4	11.7	18.9	15.0
4– 8	5.1	19.5	15.5
8–12	8.1	20.5	15.6
12–16	6.7	20.0	17.1
16–20	3.7	20.5	17.5
20–24	4.3	20.5	18.2
Total for 24 hr	39.6	119.9	98.9

a length of time exceeding the duration of these experiments. Microscopic examination of the kidneys 24 hours after ureteral ligation revealed a little dilatation of some of the tubular lumina and of a few glomerular capsules, but otherwise the kidneys appeared normal.

It was reasoned that if the kidneys inhibit protein catabolism, presumably through the mediation of a humoral agent, this substance would continue to be produced and would continue to be liberated into the blood after bilateral ureteral ligation. The rate of protein catabolism would be less, therefore, in rats subjected to bilateral ureteral ligation than in animals whose kidneys had been completely excised.

A comparison of the rates of urea formation given in Table I, and of the serum urea concentrations shown by Fig. 1, supports this hypothesis. The serum urea concentration and the rate of urea formation of the rats subjected to bilateral ureteral ligation were significantly less than the serum urea concentration and the rate of urea formation of the completely nephrectomized animals. Other factors than the presence of viable renal tissue must be concerned with inhibition of protein catabolism, however, since the rate of urea formation of the rats with ligated ureters was greater than that of mock operated controls(1) studied under the same experimental conditions (Table I.).

Summary. The serum urea concentrations and the rates of urea formation of rats subjected to bilateral ureteral ligation and of completely nephrectomized rats were compared under conditions in which adequate quantities of fluid and calories were provided before and after operation. The serum urea concentration and the rate of urea formation were less in the rats whose ureters were ligated than in the nephrectomized animals. These findings support the hypothesis that the presence of viable renal tissue is a factor concerned with inhibition of the rate of protein catabolism.

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Antibiotic Studies on Beta Hemolytic Streptococci: VI. Acquired *In vitro* and *In vivo* Resistance to Aureomycin.* (17559)

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It has not yet been reported that beta hemolytic streptococci acquire resistance to aureomycin *in vitro*. Paine, Collins, and Fin-

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land(1) reported that one strain of *Streptococcus mitis* and one strain of *Str. pyogenes* acquired no increase in resistance to aureomycin even after 60 subcultivations on antibiotic-containing medium. Harvey, Mirick, and Schaub(2) demonstrated an 8-fold increase in resistance to aureomycin by a microaerophilic streptococcus and a 16-fold increase in resistance by a *Str. faecalis*. These organisms were isolated from 2 patients during a relapse after several weeks of treatment with aureomycin for subacute bacterial endocarditis. The resistant *Str. faecalis* grew more slowly and formed smaller colonies on blood agar. The resistance of this strain was not permanent since streptococci of the original sensitivity reappeared after several transfers on blood agar.

The present study was undertaken to compare the *in vitro* and *in vivo* development of aureomycin resistance by group A, B, and C beta hemolytic streptococci, and to determine whether or not aureomycin altered the biology of the streptococcus in a manner similar to that produced by penicillin,(3-6) streptomycin,(7) and bacitracin.(8,9)

Materials and methods. Resistance to aureomycin was induced in 23 strains of beta hemolytic streptococci: 8 of group A, 8 of group C, and 7 of group B. Parent strains and their designations are the same as those used in preceding studies.(3-7) Aureomycin sensitivity was determined by the whole plate method and was taken as the maximum concentration of antibiotic that permitted good growth. Bacto heart infusion agar (Difco) containing 5% defibrinated sheep's blood and varying concentrations of aureomycin HCl (Lederle A377, Lot No. 7-8411) were used in 100 mm Petri dishes. Fresh dilutions of the yellow crystalline salt were prepared daily in physiological saline at a pH of 3.1. All antibiotic-containing blood agar plates were poured at a maximum temperature of 45°C, streaked as soon as solidified, and incubated at 37°C for 16 to 18 hours. Growth was recorded without delay after incubation to minimize any variations due to lability of the antibiotic.(1,10) To induce resistance *in vitro* 40 subcultivations were made on media containing aureomycin by the method previously described.(7) Comparison was made of the mouse virulence of the parent strain, a control strain (parent strain after 40 serial transfers on blood agar plates containing no antibiotic), the induced resistant strain, and the resistant variant after 12 passages in normal mice by an intracerebral inoculation method.(3) To ensure equality of inocula the densities of the Todd-Hewitt broth cultures of the control organism and the variants were adjusted to that of the parent strain on a Coleman junior spectrophotometer, either by the addition of broth or by the subtraction of supernatant fluid after centrifugation. The number of viable streptococci in the adjusted broth was determined by pour plate colony counts.

A standard strain of beta hemolytic streptococcus (C203 MV), a group A type 1 organism, was passed serially 25 times in mice and 30 times in embryonated hens' eggs protected with varying concentrations of aureomycin in an effort to induce aureomycin resistance *in vivo*. The method employed was essentially the same as that used to induce penicillin resistance(6) but with the following modifications: 1) neopeptone Todd-Hewitt broth cultures were adjusted to comparable densities spectrophotometrically for each passage in both the mouse and egg series; 2) the streptococci were given intraperitoneally in mice in 0.2 ml amounts; 3) mouse virulence was de-

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TABLE I.
Aureomycin Sensitivity of Group A, B, and C Beta Hemolytic Streptococci Isolated from December 7, 1948 to March 22, 1949.

	Group A strains		Group B strains		Group C strains	
μg/ml	No.	%	No.	%	No.	%
.02	17	8.3				
.05	105	51.5	5	22.7	22	45.8
.1	79	38.7	16	72.7	21	43.7
.2	3	1.5	1	4.6	3	6.3
.3					1	2.1
.5					1	2.1
Total	204	100.0	22	100.0	48	100.0

TABLE II.
Aureomycin Resistance Induced in Group A, B, and C Beta Hemolytic Streptococci by Subcultivation 40 Times on Aureomycin Medium.

Group	Strain	Initial aureomycin sensitivity,* μg/ml	Final aureomycin sensitivity,* μg/ml	Fold change
A	S1	.5	1.	2
	S2	.5	3.	6
	S3	.5	3.	6
	S4	.5	3.	6
	S5	.5	2.5	5
	S6	.5	3.	6
	U5	.5	4.	8
	U6	.3	3.	10
B	T5	.08	1.	12
	T7	.08	1.	12
	T8	.08	2.	25
	T9	.08	2.	25
	T10	.08	2.	25
	T11	.08	1.	12
	T12	.08	1.	12
C	U1	.1	1.	10
	U2	1.0	60.	60
	U3	1.0	20.	20
	U7	.1	4.	40
	U8	.1	3.	30
	U9	.1	4.	40
	U10	.5	5.	10
	U11	.1	2.	20

* Aureomycin sensitivity is defined as the maximum concentration of aureomycin in the medium permitting good colonial growth.

termined separately for each mouse passage by intraperitoneal modification of the previously described technic; 4) the dosages of aureomycin in mice were 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mg; in eggs they were from 0.1 to 2.0 mg per egg. *In vitro* sensitivities were performed at intervals by the whole plate technic.

Results. IN VITRO. A. *Initial Aureomycin Sensitivity.* Table I lists the aureomycin sensitivity of 274 recently isolated beta hemolytic streptococci of groups A, B, and C. The sensitivity varied from 0.02-0.5 μg/ml. The range of sensitivity within groups widened in the order C greater than A greater than B.

B. *Acquired Resistance Aureomycin-containing medium.* Table II summarizes the aureomycin resistance induced in 23 strains of group A, B, and C beta hemolytic streptococci after 40 serial subcultivations on aureomycin medium. The minimum increase in resistance was 2-fold in a group A organism, and maximum increase was 60-fold in a group C organism. The group B strains were the

TABLE III.

In vitro Sensitivities of Aureomycin-Resistant Beta Hemolytic Streptococci After Transfer on Control Medium or Passage Through Mice.

Group	Strain	Parent, µg/ml	Resistant variant, µg/ml	Sensitivity after transfer on control medium, µg/ml				Sensitivity after 12 normal mouse passages, µg/ml
				25 × s	50	75	100	
A	S1	.5	1	1	1	1	1	
	S2	.5	3	3	3	3	1	1.8
	S3	.5	4	3	1	1	1	4.0
	S4	.5	3					3.0
B	T5	.08	1	1	1	1	1	1.5
	T7	.08	1					1.0
	T8	.08	2					2.0
	T10	.08	2	1	1	1	1	
	T11	.08	1	1	1	1	1	
C	U1	.1	1	1	1	1	1	0.5
	U2	1.0	60	50	7	7	7	3.0
	U3	1.0	20	30	3	3	3	8.0

most uniform in their rate of development of resistance. By group, the rate of development of resistance was A less than B less than C.

C. *Acquired Resistance on Control Medium.* All 23 parent organisms were subcultured serially on control medium (aureomycin-free blood agar). Dissociation was minimal and no changes in hemolysis were observed. After 40 transfers, no significant increase in aureomycin resistance was discernible.

D. *Loss of Acquired Resistance.* Three aureomycin-resistant strains of group A, 3 of group B, and 3 of group C streptococci were subcultured serially 100 times on aureomycin-free blood agar plates in an attempt to restore their original sensitivity. The organisms were tested on the 25th, 50th, 75th, and 100th transfer by the whole plate method. Only the 2 most resistant group C strains (U_2 and U_3) lost 5/6ths of the acquired resistance after the 50th passage on plain blood agar plates. Neither, however, returned to its original level of sensitivity. These values, recorded in Table III, remained unchanged over an 8-month period of storage in broth at 4°C. Three strains each of group A, B, and C streptococci after acquiring aureomycin resistance were passed intracerebrally 12 times in normal mice and their *in vitro* sensitivities determined by the whole plate technic. Only the 2 most resistant group C strains (U_2 and U_3) showed a significant decrease in resistance (Table III).

E. *Change in Virulence.* Only 3 strains of streptococci, the U_2 and U_3 group C and the S_2 group A strains, showed an appreciable loss of mouse virulence after acquiring aureomycin resistance. In general, the behavior of the control strains was similar to that of the parent organisms. In 5 of 6 group A, B, and C strains, the virulence of the resistant variant was increased over that of either the parent or resistant organism by 12 intracerebral passages in normal mice. These results are given in Table IV. The number of viable streptococci in cultures of the parent and resistant strains was essentially the same.

F. *Antigenic and Hemolytic Changes.* The 23 beta hemolytic streptococci were regrouped by the Lancefield technic after acquiring aureomycin resistance. In only 1 resistant group B organism, the T_5 strain, were we unable to demonstrate the group-specific precipitinogen. Transient conversion from beta to alpha hemolysis occurred on concentrations of aureomycin which limited growth in several group A, B, or C organisms. These strains, when growing on control medium, had the normal beta type of hemolysis. Two group C organisms which produced an area of greening beyond the clear zone, showed an exaggeration of this color when growing on low concentrations of aureomycin. This greenish hemolytic aura completely disappeared leaving only a clear beta zone as the inhibiting concentration of aureomycin was reached.

G. *Enzymatic Alterations*. Early results of comparisons of antigenic and enzymatic alterations produced in the same beta hemolytic streptococci by the separate induction of penicillin-, streptomycin-, bacitracin-, and aureomycin-resistance have been reported from this laboratory.(9) Aureomycin-resistant organisms, in general, showed a less altered metabolism, as measured by their streptolysin-S, proteinase, streptokinase and ribonuclease production, than did either penicillin- or streptomycin-resistant streptococci. No significant change in streptolysin S activity accompanied induced aureomycin resistance in 5 group A and 3 group C organisms surveyed. The 2 group A strains that produced proteinase showed unaltered enzyme activity after induced aureomycin resistance. However, reduced streptokinase production was demonstrated in 1 of 3 group A and 1 of 2 group C resistant variants when compared with their controls. Ribonuclease activity was lessened in 2 of 7 group A streptococci by transfer on aureomycin medium.

IN VIVO. A. *Acquired Resistance in Protected Mice or Embryonated Eggs*. The C203 MV strain of beta hemolytic streptococcus developed no *in vivo* aureomycin resistance after 25 serial mouse passages, nor was there a change in the *in vitro* sensitivity at the end of these passages. The PD_{50} for aureomycin and LD_{50} for the 1st and 25th passages respectively were essentially the same. The sensitivities of the parent, intermediate and final strains as determined by the whole plate technic were identical. Similarly, the C203 MV strain showed no acquisition of resistance to aureomycin through 30 egg passages, as measured by the successive amounts of aureomycin needed to sterilize the eggs and by the change in *in vitro* sensitivity of the passaged organism.

Discussion. Resistance to aureomycin may be induced in beta hemolytic streptococci by a technic similar to that used for penicillin or streptomycin. Many subcultivations are necessary on sub-inhibitory concentrations of the drug to produce a detectable change in the sensitivity of any of the streptococci employed. The magnitude and rate of the changes are quite comparable to those previously seen for penicillin.(3,5) Failure to demonstrate the development of aureomycin resistance ***in vivo*** in a group A strain was obtained also with penicillin.(6) Both the ***in vivo*** and ***in vitro*** results suggest that the development of aureomycin resistance in beta hemolytic streptococci will not be of clinical importance if an analogy between laboratory studies and clinical results for penicillin and aureomycin is valid. To date, penicillin resistance by beta hemolytic streptococci has not been of clinical importance. This had been predicted by several laboratory studies(11,12,3,6) on induced resistance. Aureomycin appears to produce alterations in the biology of streptococci less frequently than does streptomycin or penicillin. The mouse virulence is only infrequently decreased, the group antigenicity is unchanged, and the enzyme systems are only occasionally inhibited.

Summary. 1. The aureomycin sensitivity range of 274 recently isolated group A, B, and C beta hemolytic streptococci was 0.02-0.5 μg/ml.

2. Aureomycin resistance was induced in 23 strains of group A, B, and C beta hemolytic streptococci by 40 serial subcultivations on aureomycin medium. The minimum increase in resistance was 2-fold in a group A organism, and maximum increase was 60-fold in a group C organism.

3. Serial transfer on control medium or passage through normal mice partially restored aureomycin sensitivity to resistant variants.

4. Mouse virulence was decreased in only the 2 group C organisms which had shown the highest degree of aureomycin resistance.

5. In only 1 group B resistant variant was the group specificity lost after the development of aureomycin resistance.

6. Growth on maximum concentrations of aureomycin stimulated transient conversion from clear to green hemolysis in organisms normally producing only a clear beta zone. Conversely, group C organisms with normal

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TABLE IV.
Mouse Virulence of Group A, B, and C Beta Hemolytic Streptococci with Acquired Aureomycin Resistance.

		Initial LD_{50}		LD_{50} after 40 transfers on			LD_{50} of resistant strains after 12 mouse passages
				Control medium	Aureomycin medium		
Group	Strain	Dilution*	No. organisms†	Dilution*	Dilution*	No. organisms†	Dilution*
A	S2	$10^{-4.8}$	1.6×10^2	$10^{-2.8}$	$10^{-1.5}$	3.0×10^5	
	S3	$10^{-4.5}$	2.0×10^2	$10^{-3.7}$	$10^{-3.0}$	8.4×10^3	
	S4	$10^{-4.3}$	3.7×10^2	$10^{-3.2}$	$10^{-3.0}$	4.5×10^3	$10^{-6.2}$
B	T5	$10^{-3.0}$	1.9×10^4	$10^{-4.8}$	$10^{-2.5}$	4.3×10^4	$10^{-7.0}$
	T7	$10^{-4.0}$	6.3×10^2	$10^{-5.3}$	$10^{-4.0}$	5.9×10^2	$10^{-3.2}$
	T8	$10^{-4.0}$	7.8×10^2	$10^{-6.3}$	$10^{-4.5}$	8.5×10^2	$10^{-7.0}$
C	U1	$10^{-1.5}$	2.1×10^5	$10^{-2.5}$	$10^{-1.3}$	1.0×10^6	$10^{-3.6}$
	U2	$10^{-6.0}$	9.3×10^1	$10^{-4.0}$	10^{-0}	2.2×10^7	
	U3	$10^{-2.5}$	5.8×10^4	$10^{-2.8}$	$10^{-1.2}$	2.0×10^6	$10^{-5.7}$

* The LD_{50} is expressed in terms of the dilution of the whole broth culture after it had been adjusted in density to that of its corresponding parent strain.

† The LD_{50} is expressed in terms of the number of viable streptococci inoculated.

green hemolytic characteristics lost their green hemolytic zones when grown on maximum aureomycin concentrations.

8. Reduction in streptokinase production occurred in 2 of 5 group A and C resistant strains and diminished ribonuclease activity in 2 of 7 group A resistant variants. No change in streptolysin S or proteinase activity was observed.

9. The C203 MV strain of group A streptococcus failed to acquire aureomycin resistance after repeated exposures to sublethal amounts of the antibiotic either in mice or embryonated hens' eggs.

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VII. Vitamin B_{12} and the Intrinsic Factor. (17560)

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Soon after the discovery by Minot and Murphy(1) of the effectiveness of liver in the dietary treatment of pernicious anemia, it was shown that foods such as beef muscle contain a heat-stable substance, designated the extrinsic factor,(2) which produces little or no response when ingested by a pernicious anemia patient unless normal gastric juice is also administered with it. The activity of gastric juice has been ascribed to the so-called intrinsic factor; gastric juice alone has no oral hematopoietic power.(3) Over a period of years the relationship between the extrinsic and intrinsic factors has been the subject of considerable clinical experimentation by Castle and his associates, and other investigators. Recent findings on this subject are as follows: Vitamin B_{12}, active parenterally at the microgram level, has been found to be essentially inactive when administered orally

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at a dosage level of 5 μg(4,5) daily. However, when a 5-μg daily dose of vitamin B_{12} was administered orally together with normal human gastric juice, a satisfactory hematopoietic response was observed. It has been suggested(4) that the food (extrinsic) factor may be identical with or closely related to the antipernicious anemia principle in liver, which is itself presumably identical with vitamin B_{12}. These investigators further suggest that gastric juice is necessary for optimal utilization of the small amounts of vitamin B_{12} in foods by promoting more effective absorption; (4) a direct reaction between extrinsic and intrinsic factors does not necessarily occur. Similar results and conclusions have been reported by others.(6,7) Prior to this interpretation, much work had been based upon the possibility of a chemical reaction between the two factors to give another substance which was the effective one.(3)

The purpose of the present work was to study the possible chemical reaction between vitamin B_{12} and normal human gastric juice when incubated under several conditions for such enzyme or other reactions, and to determine whether the extrinsic factor of beef muscle is hematopoietically active when administered by the parenteral route.

Experimental. Samples of residual gastric juice of fasting individuals* were collected in the morning; then, 0.5 mg of histamine acid phosphate was administered intramuscularly and the gastric juice was collected for an additional thirty minutes. The samples were chilled immediately, filtered through gauze, and stored frozen in dry ice. The samples were assayed(8) for LLD activity; (9)† the results are in Table I. In their early studies, Castle and Townsend(2) gave to pernicious anemia patients daily oral doses of gastric juice in quantities up to 300 ml without obtaining positive responses. Based on an average value of 100 u./ml, it is evident that this volume of gastric juice probably contained only about 3 μg of vitamin B_{12}, or its equivalent in microbiological activity. Such an amount of vitamin B_{12} as a single dose is ineffective orally, but effective parenterally.(10) In 1932, it was shown(11) that a vacuum-concentrate of 450 ml of normal gastric juice produced a striking hematopoietic effect when administered intramuscularly; this result can be interpreted now as due to the presence of an adequate amount of vitamin B_{12}-like activity in the concentrate. It was shown later,(12) that hematopoietic responses were obtained by parenteral injection of similar concentrates of the gastric juice of swine, dogs, and cattle.

TABLE I.
LLD Activity of Gastric Juice.

Individual	Fasting gastric juice, u/ml	Histamine induced gastric juice, u/ml
1	97	23
2	48	42
3	66	52
4	100	68
5	126	81
6	103	100
Avg	108	73

The stability of vitamin B_{12} when dissolved in gastric juice was examined. Crystalline vitamin B_{12} was dissolved in filtered gastric juice and the solutions were incubated at 28-30°C. The solutions were examined for

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* Courtesy of S. M. Hoch, M.D., Plant Health Department, Merck and Co., Inc.

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† The LLD activity of a given preparation is a measure of its growth-promoting effect for *L. lactis* Dorner. Vitamin B_{12} has an LLD activity of 11×10^6 units/mg.

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TABLE II.
Activity of Solutions of Vitamin B_{12} in Gastric Juice.

	Fasting gastric juice, u × 10^6/mg		Histamine-induced gastric juice, u × 10^6/mg
Time in hr	pH 2	pH 7	pH 3
.25–.50	12	12.5	11
3	13	16	
6	11	10	13
22	9.3	11	13

changes in absorption spectrum and microbiological activity. Both residual gastric juice and histamine-stimulated gastric juice were used. The absorption spectra of the solutions showed no changes during incubation at pH 2. There was no evidence of a change in the microbiological activity of the solution at pH 2, 3, and 7 as summarized in Table II.

A 16.4-mg sample of crystalline vitamin B_{12} was dissolved in 25 ml of gastric juice obtained from a fasting individual. The solution (pH 2.3) was allowed to stand eighteen hours at 37° then seven hours at pH 7.2. At the end of the incubation period, vitamin B_{12} was recovered from the mixture. Because of large amounts of solids in gastric juice only a portion of the vitamin B_{12} was recovered in crystalline form. The refractive indices of the crystals which were obtained were in satisfactory agreement with those reported(13) for crystalline vitamin B_{12}.

An LLD-active concentrate was prepared from 25 pounds of beef muscle by the following sequence of steps: extraction of ground lean chuck with hot water, precipitation of inert matter from the concentrated extract by 70% ethanol, precipitation of an active fraction from aqueous solution by saturation with ammonium sulfate, dialysis, and extraction of the active fraction from the diffusate with o-cresol. The final product (2.28 g), after lyophilizing from aqueous solution, was a nearly white solid having an activity for *L. lactis* of about 500 μ/mg. About 40% of the original activity was recovered. This LLD-active concentrate from beef muscle was found by Hall(14) to be hematopoietically active in 3 cases of pernicious anemia when administered parenterally in a daily dose of about 20 mg. The amount given daily had a microbiological activity equivalent to about 1 μg of vitamin B_{12}. Thus, the extrinsic factor in a concentrate from beef muscle elicits a hematopoietic response on a microgram-basis very similar to that of vitamin B_{12}.

Summary. Human gastric juice contains small amounts of vitamin B_{12} or its equivalent as assayed by *L. lactis*. Crystalline vitamin B_{12} was added to gastric juice and the solutions were incubated at pH 2, 3 and 7; there were no observed changes in the absorption spectra or in the microbiological activity over a 22-hour period. Again, crystalline vitamin B_{12} was added to gastric juice, and after incubation at pH 2 and 7 at 37°, much of the vitamin B_{12} was recovered. No evidence was found for a chemical reaction between vitamin B_{12} and the intrinsic factor which results in a gross structural change in vitamin B_{12}. A possible reaction between vitamin B_{12} and the intrinsic factor in which there was a very small structural change of the large vitamin B_{12} molecule, and which could produce as little as 5 μg of orally active product is being investigated. A concentrate prepared from beef muscle having vitamin B_{12}-like microbiological activity was found to be hematopoietically active when administered by the parenteral route to pernicious anemia patients; thus, the extrinsic factor acts like vitamin B_{12}. These results support the previously derived hypothesis that the effect of the intrinsic factor is due to its ability to promote the absorption of the small amounts of vitamin B_{12} or its equivalent which occur in natural foods. The mechanism of this enhancement is being studied further.

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Further Studies on Modification of Sensitivity to X-rays by Cysteine.
(17561)

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In a previous communication(1) it was shown that cysteine greatly reduced the sensitivity of rats to lethal amounts of x-rays delivered at high dose rates, provided that the amino acid was given before the exposure. In contrast to the protective influence of cysteine was the finding that cystine did not alter survival of the irradiated animal. We should like to present in this paper further data concerning the time course of the cysteine protection as well as a comparison of the influence of certain substances on radio sensitivity.

Methods. The methods were the same as those reported earlier.(1) Control and experimental animals were irradiated simultaneously, caged together, and otherwise similarly handled. Male and female rats (Sprague-Dawley) weighing 150-250 g and female mice (CF-1) weighing 20-25 g were employed. Cysteine was administered as a 10% solution of the hydrochloride neutralized to pH 7-8 with 2N sodium hydroxide. The cysteine dose was 950 mg/kg intravenously or 1900 mg/kg orally. Although the oral dose is well tolerated, the smaller intravenous dose is near the maximal tolerated amount and must be injected slowly to prevent acute death in some of the animals.* Cysteine was injected into a tail vein or given by stomach tube at one of the following times relative to irradiation: 5 minutes, 30 minutes, 1 hour, 6 hours, or 24 hours before or 5 minutes after. Irradiated controls received comparable volumes of 5% sodium chloride intravenously or orally.

Comparison was also made of the influence on sensitivity to total-body X radiation of cystine (I.V. and oral), glutathione (I.V. and oral), ascorbic acid, sodium sulfide, and colloidal sulfur (all I.V.). These substances were administered within an hour before the exposure. In most instances, two doses of each substance were used, the maximal tolerated dose and one-half of this. In the case of cystine and methionine, dosage was determined largely on the basis of solubility. Colloidal sulfur was prepared by dissolving recrystallized sulfur in hot 95% ethyl alcohol, addition of cold distilled water, and subsequent removal of the alcohol by heating. Rats received 800 r total-body X radiation in a single exposure, mice 600 r. The radiation factors were 250 kv, 15 ma, 0.5 mm Cu, and 3.0 mm Bakelite filters, 26.5 cm target distance, 1.5 mm Cu half-value layer, and 210-220 r/minute dose rate. In a few experiments rats and mice were given 800 r and 600 r, respectively, at 15-20 r/minute in order to compare cysteine protection against x-rays delivered at high and low intensities. Survival at 28 days post irradiation was the criterion selected to evaluate protection.

Results. Rather comparable protection is observed when cysteine is administered intravenously to rats 5 minutes or 1 hour before X irradiation (90% survival of 114 cysteine irradiated, 10% survival of 117 irradiated controls). Injection 5 minutes after or 6 or 24 hours before is without influence on survival of the irradiated rat. These findings are presented in Fig. 1. Acute radiation deaths occur largely from 1 to 3 weeks after the exposure with a peak in the second week. The few deaths occurring in the groups given cysteine within an hour prior to irradiation are scattered over this interval. Cysteine administered orally also confers significant protection to rats and mice when it is given before the exposure. However, oral administration is not as efficient as intravenous even

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1. Patt, H. M., Tyree, E. B., Straube, R. L., and Smith, D. E., *Science*, 1949, v110, 213.

* Recent experiments on x-ray-cysteine dosage relationships have revealed that lower doses of cysteine can afford rather equivalent protection to rats against 800 r. (Smith, D. E., Patt, H. M., Tyree, E. B., and Straube, R. L., American Physiological Society Fall Meeting, Augusta, Georgia, September 14-16, 1949).

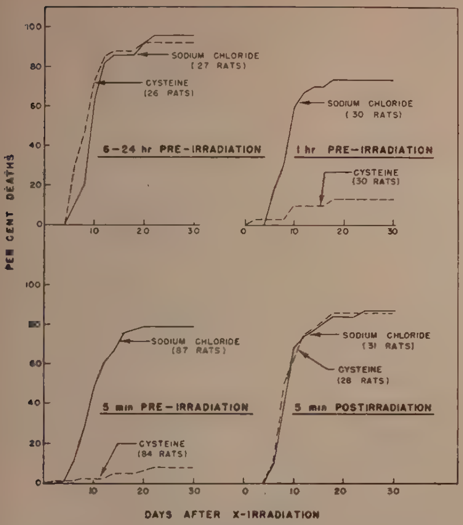

FIG. 1.

Influence of time of injection of cysteine (950 mg/kg I.V.) on sensitivity of rats to total-body X irradiation with 800 r.

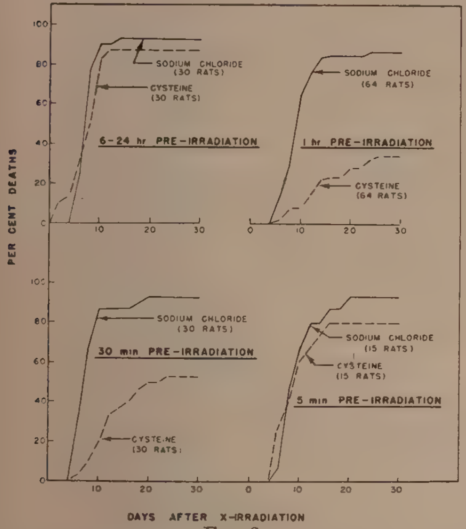

FIG. 2.

Influence of time of oral administration of cysteine (1900 mg/kg *per os*) on sensitivity of rats to total-body X irradiation with 800 r.

though the oral dose is twice the latter. The effective interval in the case of cysteine administered by stomach tube is 30 to 60 minutes before irradiation. Since cysteine protection is similar in irradiated rats and mice, only data obtained in the former are presented (Fig. 2). The cysteine effect is apparently independent of x-ray dose rate, at least within wide limits. A similar reduction in mortality is obtained when the amino acid is given to mice and rats exposed to 600 r and 800 r, respectively, at either 15 to 20 r/minute or 210 to 220 r/minute. At the former dose rate, 69% of 45 mice given cysteine orally survive as compared with 28% of 47 irradiated controls. When cysteine is administered intravenously to rats before exposure at the low dose rate, 84% of 19 cysteine irradiated survive as compared with 16% of 25 irradiated controls. This protection is of the same order as presented in Figs. 1 and 2 for high dose rate exposures.

The influence of related substances on the sensitivity of rats to x-rays is summarized in Table I. Cystine is not effective either intravenously or orally. Glutathione can reduce x-ray toxicity in rats but only when it is given intravenously before the exposure.† The reduction in sensitivity to x-rays by glutathione is roughly two-thirds that observed with cysteine. In contrast to the protective influence of cysteine by the oral route, glutathione is without effect in mice and rats when administered orally (mice, 5000 mg/kg glutathione *per os*, 50% survival of 20; irradiated controls, 40% survival of 20—rats, see Table I). Methionine, ascorbic acid, and sodium sulfide in the dosages used failed to alter survival of rats after irradiation. Colloidal sulfur appeared to reduce radiation toxicity in one experiment but not in another.

Discussion. Studies on the time course of cysteine protection against total-body X irradiation reveal that the amino acid must be given before the exposure. This suggests that the reactions leading to morbidity are complete when the irradiation is terminated and that these reactions are apparently not reversible. It is, of course, possible that these reactions could be reversed if the cysteine were given quickly enough, perhaps

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† In a personal communication, E. P. Cronkite (Naval Medical Research Institute) has indicated that he has obtained similar protection in mice given glutathione subcutaneously before X irradiation.

TABLE I.
Influence of Related Substances on Sensitivity of Rats (Sprague-Dawley) to 800 r X-rays.

Treatment group	Dose, mg/kg	Route*	No. of rats	% survival, wk after irradiation			
				1	2	3	4†
Control	—		48	67	27	21	21
Glutathione	625	I.V.	30	93	73	67	*63*
Control	—		14	93	43	21	21
Glutathione	900	I.V.	14	93	64	64	*64*
Control	—		16	31	6	0	0
Glutathione	1250	I.V.	10	50	40	40	*40*
Control	—		30	30	7	7	7
Glutathione	2000	Oral	29	34	7	3	3
Control	—		27	52	22	15	15
Cystine	280	I.V.	22	68	18	18	18
Control	—		15	27	0	0	0
Cystine	2500	Oral	13	23	8	8	8
Control	—		33	58	27	21	21
Methionine	255	I.V.	16	56	31	25	25
Control	—		15	40	20	20	20
Ascorbic acid	500	I.V.	15	53	13	7	7
Control	—		15	47	20	20	20
Ascorbic acid	1000	I.V.	15	53	13	13	13
Control	—		15	87	7	7	7
Sodium sulfide	2.5	I.V.	15	73	20	7	7
Control	—		15	67	27	13	0
Sodium sulfide	5.0	I.V.	15	73	13	7	7
Control	—		15	87	27	0	0
Colloidal sulfur	17.5	I.V.	12	100	67	42	*42*
Control	—		15	93	20	20	20
Colloidal sulfur	20	I.V.	14	79	29	29	29

* All I.V. injections were made 5 minutes before irradiation; oral administration, 1 hour before.

†Italic figures are significantly different ($p < .05$) from the 4-week survival of the appropriate irradiated controls.

within seconds, after the exposure. Less than 10% of the intravenously injected dose can be accounted for in blood as cysteine and cystine within several minutes after its administration.(2) The blood level of cysteine at the time of irradiation is, therefore, probably not critical since comparable protection is obtained when cysteine is injected 5 minutes or 1 hour before the exposure. Differences in the protection afforded by the intravenous and oral routes when the amino acid is given 5 minutes before irradiation are apparently a reflection of the time required for absorption of the material from the gastro-intestinal tract and its distribution by the blood. Under any circumstances, oral administration of cysteine is not as efficient as intravenous even though the oral dose is twice the latter.

It was hoped that some insight into the mechanism of the cysteine protection might be forthcoming from a consideration of the influence of certain related substances on sensitivity to radiation. The term, related substances, is used in a broad sense and is not meant necessarily to imply either structural or pharmacological similarity. It is significant that cystine is not effective either intravenously or orally. The related amino acid, methionine, is also without influence on radiosensitivity. On the other hand, glutathione, the tripeptide containing cysteine, can reduce x-ray toxicity when it is given intravenously before the exposure. When glutathione is given by stomach tube, however, survival of irradiated rats and mice is unchanged even though the oral dose is equivalent to the effective oral dose of cysteine in sulfhydryl content. Our data do not permit comparison of the relative effectiveness of intravenously administered cysteine and glutathione on an equimolar basis. The maximum tolerated glutathione dose (1250 mg/kg I.V.)

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2. Patt, H. M., Smith, D. E., Straube, R. L., and Tyree, E. B., unpublished observations, 1949.

is equivalent to approximately half of the cysteine dose used for comparison (950 mg/kg I.V.) in terms of sulfhydryl content.

Sodium sulfide and colloidal sulfur were included to test further the possible importance of sulfur in the protection afforded by cysteine against x-rays. The former was ineffectual, while the latter gave equivocal results. Ascorbic acid selected because of its reducing properties and its presumed importance in biologic oxidation-reductions failed to alter survival of the irradiated rat. A sulfhydryl group is the most evident distinguishing feature between the substances which definitely protect and those which do not. It should be borne in mind, however, that all sulfhydryl-containing materials may not necessarily protect and that other reducing substances which are properly distributed, temporally and spatially, may.

Since cysteine, but not cystine, is effective in altering sensitivity to radiation, one may speculate that the cysteine acts by protecting certain cellular components from oxidation by the products of irradiated water. This could be accomplished indirectly by the reduction of one or more of the critical links in the metabolic chain or directly by neutralization of the oxidizing agents presumably produced by radiation. Barron and co-workers(3) have reported that small doses of x-rays inactivate sulfhydryl enzymes *in vitro* by oxidation of their -SH groups (reversible inhibition) and large doses by denaturation of the protein moiety (irreversible inhibition). Non-sulfhydryl enzymes may be irreversibly inhibited by still larger amounts of x-rays, primarily by protein denaturation. If enzyme inactivation of the types reported by Barron is an important factor leading to death of the irradiated animal, it would appear from the above considerations and our data on the time course of the cysteine protection that the inactivation is of the irreversible non-specific type resulting from protein denaturation.

Summary. Cysteine greatly reduces the sensitivity of rats and mice to lethal amounts of x-rays delivered at either high or low dose rates provided that the amino acid is given before the exposure. Rather comparable protection is obtained when cysteine is injected intravenously in rats immediately or 1 hour before irradiation with 800 r (90% reduction in the 28-day mortality). Injection immediately after exposure or 6 or 24 hours before is without influence. Cysteine also improves survival significantly when it is given orally 30 to 60 minutes prior to the irradiation. Glutathione (I.V. but not oral) can also diminish radiation toxicity. Cystine, methionine, ascorbic acid, and sodium sulfide do not alter survival of the irradiated animal. Results with colloidal sulfur are equivocal. The possible significance of these observations is discussed.

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3. Barron, E. S. G., Dickman, S., Muntz, J. A., and Singer, T. P., *Jour. Gen. Physiol.*, 1949, v32, 537.

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Vitamin B-12 in Amino Acid Metabolism.* (17562)

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McGinnis *et al.*(1) reported that blood non-protein nitrogen content was higher in chicks deprived of animal protein factor (APF) than in normal controls, and suggested that this may have been due to a function of APF in enhancing amino acid utilization. Zucker and Zucker(2) found that non-protein nitrogen, and urea values in rat blood were increased in zoopherin deficiency, and have elsewhere related zoopherin to the animal protein factor. Norris(3) stated that evidence found by his group (not reported in detail) pointed to such a function on the part of APF. The observation of Bird *et al.*(4) that normal chicks have no appreciable requirement for APF after 8 weeks of age may further corroborate the same theory inasmuch as the period during which APF *is* required is the time of most rapid growth, and utilization of amino acids.

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* Scientific Journal Series No. 311, Colo. Agr. Exp. Station.

1. McGinnis, James, Hsu, P. T., and Graham, W. D., *Poultry Sci.*, 1948, v27, 674.

It cannot yet be stated with certainty that the effects cited, or those observed in the present work were due entirely to the vitamin B-12 contained in the APF or zoopherin preparations used. There are many indirect indications, however, that the principal activity involved probably was that of vit. B-12. Perhaps the most direct evidence is that obtained by Ott *et al.*,(5) who found that crystalline vit. B-12 exerted APF activity in chicks deprived of animal protein. They concluded that since the crystalline vitamin elicited growth responses comparable to those obtained with crude sources of APF, it is possible that vit. B-12 is identical with or closely related to this factor.

Evidence is presented herewith in more direct support of the theory that vit. B-12 functions in amino acid utilization in chicks. The term 'vitamin B-12' refers hereinafter to an activity provided in the form of the Merck and Co. APF Supplement No. 3.

Experimental. In Exp. 1 mixed single comb White Leghorn chicks from APF-depleted dams were given corn meal for the first 2 days of life, then placed on the experimental diets. Twenty-five chicks were placed in each group, according to a uniform weight distribution pattern. Feed and water were supplied *ad libitum*. The basal diet contained sodium proteinate† 25, corn meal 70, dicalcium phosphate 3, pulverized limestone 1, DL-methionine 0.3, and choline chloride 0.17%. Vitamins and minerals were added, in terms of milligrams per kilogram of diet, as follows: niacinamide 17.5; calcium pantothenate, riboflavin and 2-methyl-1,4-naphthoquinone, each 10; thiamin hydrochloride, pyridoxine hydrochloride, pteroylglutamic acid, p-aminobenzoic acid and alpha tocopherol, each 5; manganese, 27.5; iron, 10; copper, 1; cobalt, 0.1; and sodium chloride, 2500. Vitamin A and D oils were incorporated to provide 6600 I.U. of vit. A and 750 A.O.A.C. units of vit. D per kg of diet. Because certain treatments in this experiment called for the use of iodinated casein, all lots were supplemented to contain 7 mg iodine per kg diet.

In Exp. 2 single comb White Leghorn cockerels from a commercial hatchery were kept on a vit. B-12 depletion diet for 2 weeks; then placed on experimental diets. Twenty-five chicks were placed in each group, according to a uniform weight distribution pattern. The experimental basal diet was as described for Exp. 1 except that iodine supplementation was reduced from 7 to 2.2 mg per kg of diet. The latter iodine level has been found in previous work to be protective against goitre, and experimental treatments did not necessitate balancing at the higher level. The depletion diet was the same as the Exp. 2 experimental basal except that solvent process soybean meal was used at 52.5% and corn meal at 42.5% in place of sodium proteinate at 25% plus corn meal at 70%. To facilitate depletion with respect to vit. B-12,(6) this diet was supplemented with 0.05% iodinated casein (Protamone‡).

Experimental measurements were made of growth, feed consumption, thyroid weight, thyroid histology, and blood contents of: nonprotein nitrogen, alpha amino nitrogen, eryth-

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2. Zucker, L. M., and Zucker, T. F., *Arch. Biochem.*, 1948, v16, 115.

3. Norris, L. C., Verbal report at 1948 annual meeting of Poultry Science Assn., Fort Collins, Colo.

4. Bird, H. R., Marsden, S. J., Groschke, A. C., and Lillie, R. J., *Poultry Sci.*, 1948, v27, 654.

5. Ott, W. H., Rickes, E. L., and Wood, T. R., *J. Biol. Chem.*, 1948, v174, 1047.

† Derived from soybeans and kindly provided by Dr. J. W. Hayward of the Archer-Daniels-Midland Company, Minneapolis.

6. Robblee, A. R., Nichol, C. A., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 400.

‡ Through the courtesy of Dr. W. R. Graham, Cerophyl Laboratories, Kansas City, Mo.

rocytes, hemoglobin, total amino acids,(7) and 7§ individual amino acids, namely arginine, lysine, methionine, tryptophan, histidine, threonine and valine.

Blood for analysis was collected by ventricular puncture in Exp. 1, and from the carotids after decapitation in Exp. 2. In both cases potassium oxalate was used as the anticoagulant. Individual samples were retained separately until the effectiveness of anticoagulant measures could be verified. Clotted samples and those containing disproportionately large amounts of oxalate were discarded. The remaining samples were pooled according to experimental treatments, and in Exp. 1 according to sex. The pooled samples were stirred gently and subdivided for preparation of both laked(8) and unlaked(9) protein-free filtrates. The filtrates were analyzed for total non-protein nitrogen by the procedure of Koch and McMeekin,(10) and for each of the 7 amino acids by microbiological methods employing *Streptococcus faecalis*, A.T.C.C. 9790.

Of all the various measures taken only growth, feed consumption, non-protein nitrogen and the specific amino acids showed meaningful variations. These are summarized in Table I. The blood contents shown are those obtained by the assay of unlaked protein-free filtrates. Values were obtained by the use of laked filtrates also; but are not reported in detail since this technic provided relatively poor sensitivity to the experimental treatments. The variations encountered in these values show in most instances the same trends as those shown in Table I; thereby lending a degree of further corroboration.

Discussion. It is apparent from Table I that the circulating blood of the birds receiving vit. B-12 contained less non-protein nitrogen, and less of each of the amino acids measured than did the blood from birds deprived of B-12. It also is apparent that the

TABLE I.
Effect of Dietary Vitamin B-12 on Growth, Feed Utilization and Certain Blood Contents.

Exp. No.		Avg init. wt, g	Avg wt gain, g	Avg gain per g feed, g	Blood components—Avg mg % in blood							
					Total NPN	Arginine	Lysine	Methionine	Tryptophan	Histidine	Threonine	Valine
1*	Basal	41	250	0.56	20.7 (5)‡	6.6 (4)	5.8 (2)	0.77 (3)	1.56 (4)			
	Basal B-12§	41	266	0.61	18.6 (4)	6.6 (6)	5.0 (2)	0.69 (3)	1.43 (6)			
2†	Basal	97	153	0.53	19.0 (2)	6.7 (3)	7.4 (2)	0.57 (1)	1.53 (3)	1.46 (2)	4.7 (1)	5.4 (1)
	Basal B-12§	96	164	0.60	16.1 (1)	5.8 (3)	5.3 (2)	0.40 (1)	1.36 (4)	1.11 (2)	3.2 (1)	4.0 (1)

* Duration, 1st 4 weeks of life. Exp. 1 values are averages of both sexes. No sex differences were found in blood contents.

† Duration, 3rd and 4th weeks of life. Exp. 2 was performed with cockerels.

‡ Number of replicate determinations for each value shown in parentheses. Values are averages except those determined only once. Limitations were imposed by exhaustion of materials (protein-free filtrate) for analysis.

§ Merck and Co. APF Supplement No. 3, kindly provided by Dr. D. F. Green. Diet contained the equivalent of 50 μg of vit. B-12 per kg of feed.

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7. Danielson, *J. Biol. Chem.*, 1933, v101, 505.

§ Only the first 4 in the case of Exp. 1.

8. Folin and Wu, *J. Biol. Chem.*, 1919, v38, 81.

9. Folin, *J. Biol. Chem.*, 1930, v86, 173.

10. Koch and McMeekin, *J. Am. Chem. Soc.*, 1924, v46, 2066.

birds given B-12 grew more rapidly. Since better growth was obtained at lower blood levels of amino acids, it appears likely that one function of vit. B-12 is to enhance anabolic processes, which remove amino acids from the blood to form fixed tissues. More efficient utilization of blood components leading to lower blood levels could well result in reduction of renal wastage. This in turn should be reflected in greater weight gain per unit of feed: which was found to be the case in both experiments.

The high efficiency of feed utilization observed in these experiments is of interest. Mishler, Carrick and Hauge(11) reported that when a similar ration containing presumably adequate B-12 (as fish solubles) was further supplemented with 0.3% DL-methionine, the methionine gave improved growth and feed utilization. In the present studies the diets were supplemented with 0.3% of DL-methionine, and feed utilization efficiency was very high even in the absence of B-12; although still further enhanced by its presence. A practical type, high energy starter containing animal products (2.5% fish meal, 7.5% meat and bone scrap, 2.5% dried whey) has given at best about 0.45 g gain per g feed. This ration presumably contained ample B-12. Methionine supplementation may have been a requisite condition for the high feed utilization efficiency observed in the present experiments.

The blood methionine levels were unexpectedly low compared to those of the other amino acids, since examination of the amino acid composition of both chicken muscle and of the diets used showed much higher relative contents of methionine than that found in blood. Similar low methionine values (0.106 to 0.317 mg%) were reported relative to other amino acid blood levels in rats.(12) These several findings may reflect some unique relationship of methionine in metabolism, particularly as to the role of vit. B-12 in amino acid utilization.

Summary. Vit. B-12 has been shown to reduce blood levels of non-protein nitrogen and of 7 individual amino acids from the levels found in vit. B-12 deficient chicks.

Chicks given vit. B-12 grew more rapidly and utilized feed more efficiently than B-12 deficient controls, although the latter had higher blood levels of amino acids.

Vit. B-12 appears to function in metabolism by enhancing utilization of circulating amino acids for building fixed tissues.

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11. Mishler, D. H., Carrick, C. W., and Hauge, S. M., *Poultry Sci.*, 1948, v27, 263.

12. Wiss, O., *Helv. Chim. Acta*, 1948, v31, 2148.

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Action of Aureomycin and Chloromycetin on the Virus of Primary Atypical Pneumonia.* (17563)

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Recently published reports of clinical studies on the treatment of primary atypical pneumonia with aureomycin(1-4) have indicated effectiveness of this antibiotic in shortening the course of the disease. Additional evidence for the effectiveness of aureomycin against the virus of atypical pneumonia will be found in the experimental studies with cotton rats and chick embryos to be reported in this paper.

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* This work was supported in part by a grant from the Lederle Laboratories.

1. Schoenbach, E. B., and Bryer, M. S., *J. Am. Med. Assn.*, 1949, v139, 275.

2. Kneeland, Y., Jr., Rose, H. M., and Gibson, C. D., *Am. J. Med. Sc.*, 1949, v6, 41.

3. Finland, M., Collins, H. S., and Wells, E. B., *New England J. Med.*, 1949, v240, 241.

4. Meiklejohn, J., and Shragg, R. I., *J. Am Med. Assn.*, 1949, v140, 391.

The observations to be recorded also help to elucidate the etiology of primary atypical pneumonia. In 1942 a virus transmissible to chick embryos, cotton rats, and hamsters was isolated from the sputum and lungs of patients with this disease.(5) Filtration experiments indicated a maximum particle size of 180 to 250 mμ for this agent(6) and it was shown to be distinct from viruses of the psittacosis group(7) and from Q fever.(8)† Evidence for the causative relationship of this virus to primary atypical pneumonia was based on the demonstration of an increase in neutralizing antibodies associated with illness in approximately 60% of the group of cases tested(8,9) which now numbers well over a hundred. The specificity of this virus neutralization was supported by the following evidence. Acute respiratory diseases such as influenza, psittacosis, and bacterial pneumonia which could be identified by clinical or laboratory methods did not stimulate production of neutralizing antibodies for the virus.(7-9) Serum from atypical pneumonia patients did not show a significant development of neutralizing antibodies for other agents including several respiratory viruses isolated from cotton rats and hamsters.(9)

Materials and methods. Virus. Two strains of the virus of atypical pneumonia, Mac and De were used in the 26th to 30th amniotic passages. The methods of passage and of preparation and storage of the virus suspensions were the same as those detailed in previous papers. Penicillin in a concentration of 100 units per ml was added routinely for the purpose of reducing contamination. The infectious titer of these suspensions averages approximately 10^{-2} in cotton rats and 10^{-3} to 10^{-4} in chick embryos.(6)

Antibiotics. Aureomycin was dissolved in saline containing 0.05 M phosphate buffer. Solutions were made up fresh daily and injected as soon as possible except that in some instances the unused portion of the solution remaining in the vial was stored at −70°C overnight and used up the next day. Chloromycetin was dissolved or suspended in plain physiological saline, complete solution being obtained only in concentrations of 5 mg per ml or less.

Experiments in cotton rats. Cotton rats of the species *Sigmodon hispidus hispidus* were obtained from a single source. The animals used in these experiments were 6 to 8 weeks of age and weighed between 60 and 80 g. Their diet consisted of Purina dog chow and water. In each experiment animals of a single shipment as uniform as possible in age and weight were divided between treated and control groups. The cotton rats were inoculated intranasally under ether anesthesia with lightly centrifuged 20% suspensions of chick embryo tissue. The antibiotics were injected once daily by the intraperitoneal route in a volume of 0.5 to 1.0 ml of solution or suspension starting 24 hours after the virus or later. The experiments were terminated 10 days after the inoculation of virus at which time all animals were sacrificed, autopsied, and the extent of the pulmonary lesions recorded. The usual system of recording lesions by gross pathology and of calculating lesion scores was used with certain modifications. Lesions involving ½ to ¼ of the total volume of lung tissue were recorded as 2, those involving ¼ to ⅛ of the lung as 1, those involving less than ⅛ of the lung as ½. Lesions less than 1 millimeter in diameter were recorded as ± and may be considered to have doubtful significance. In calculating the lesion scores, however, such ± lesions were arbitrarily assigned a score of ¼. The lesion scores in per cent were obtained as usual by adding together the scores for the animals in a group and dividing by 5 times the total number in the group. In representing the infection ratio of a group of animals (number having pulmonary lesions over the total inoculated) the

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5. Eaton, M. D., Meiklejohn, G., and van Herick, W., *J. Exp. Med.*, 1944, v79, 649.

6. Eaton, M. D., Meiklejohn, G., van Herick, W., and Corey, M., *J. Exp. Med.*, 1945, v82, 317.

7. Eaton, M. D., PROC. SOC. EXP. BIOL. AND MED., 1945, v60, 231.

8. Eaton, M. D., and van Herick, W., *Am. J. Hygiene*, 1947, v45, 82.

† See footnote page 93 and discussion on pages 92 and 93 of reference 8.

9. Eaton, M. D., van Herick, W., and Meiklejohn, G., *J. Exp. Med.*, 1945, v82, 329.

TABLE I.
Effect of Dosage of Aureomycin in Cotton Rats, Strain De.

Exp. No.	Aureomycin dosage		No. of animals with pulmonary lesions of					Avg L.S.†	Infection ratio
	mg	Started at, hr	2	1	½	±	0		
1	1	24	0	1	0	1	12	1.8	1/14
	S*	”	1	5	3	2	5	11.3	9/16
2	1	”	0	0	0	0	8	0	0/8
	.5	”	1	2	1	0	3	12.8	4/7
	S	”	2	2	1	0	2	18.5	5/7
3	.5	”	0	0	1	2	4	2.9	1/7
	S	”	0	3	1	1	1	10.8	4/6
4	.5	”	0	0	2	1	4	3.6	3/7
	.2	”	0	3	2	2	0	12.8	7/7
	0	—	0	3	4	0	0	14.3	7/7
5	.2	24	0	0	2	2	3	4.2	2/7
	S	”	0	1	1	1	4	5.0	2/7

* S indicates that buffered saline was inoculated by the intraperitoneal route in place of the antibiotic. 0 indicates no inoculation.

† L.S. denotes pulmonary lesion score. See section on *Materials and Methods.*

± lesions were not counted as indicative of infection.

Experiments in chick embryos. Chick embryos inoculated by the amniotic route with the atypical pneumonia virus received aureomycin into the yolk sac. Embryos dying on the 1st to 6th day after inoculation constituted about 20 to 30% of the total groups of treated or control eggs and these were discarded. Surviving embryos were harvested 7 days after inoculation. The pooled lungs, trachea, and amniotic membranes from all eggs of the treated and control groups were made into 20% suspension. This material from the two groups of eggs was then inoculated intranasally into cotton rats to test for the presence of virus.

Results with Aureomycin in cotton rats. Determination of minimal effective dose. A series of experiments was done with aureomycin in daily doses of 0.2 to 1.0 mg per cotton rat starting treatment 24 hours after inoculation of the virus. The results are presented in Table I. Twenty-two animals were treated with aureomycin at a dosage of 1.0 mg. (Exp. 1 and 2). Only one of these developed a significant pulmonary lesion while 14 of the 21 control animals in these two experiments had lesions. The average lesion scores in the treated animals were reduced by approximately 90% as compared with the controls.

When the dosage was reduced to 0.5 mg per cotton rat daily (Exp. 2, 3 and 4) significant effects were also observed in two experiments, but a higher proportion of the animals developed pulmonary lesions and in Experiment 2 no significant difference between the control and treated group was obtained. In the 3 experiments together 8 of the 21 animals receiving a daily dose of 0.5 mg of the antibiotic developed pulmonary lesions while 16 of the 20 controls were positive. Two experiments (4 and 5) were done with aureomycin at a dosage of 0.2 mg. No difference in the incidence of infection between the treated and control animals was observed in either experiment, although the lesion score in the treated animals was slightly lower than that of the controls. The results indicate that minimal effective dose of aureomycin is approximately 0.5 mg. The average body weight of the cotton rats used in these experiments varied from 54 g in Experiment 2 to 69 g in Experiment 4 thus giving a range of 7.3 to 9.3 mg/kg of body weight at this critical dosage level.

Effect of treatment at increasing time after inoculation. In a series of 5 experiments aureomycin at a dosage of 2.0 mg per cotton

TABLE II.
Effect of Time of Starting Treatment with Aureomycin in Cotton Rats.

Exp. No.	Strain	Aureomycin dosage		No. of animals with pulmonary lesions of					Avg L.S.	Infection ratio
		mg	Started at, hr	2	1	½	±	0		
6	Mac	2	24	0	0	0	2	7	1.1	0/9
		S	”	1	2	3	1	3	9.5	6/10
7	De	2	48	0	0	1	1	8	2.5	1/10
		S	”	0	3	4	1	2	10.5	7/10
8	”	2	72	0	0	0	0	12	0	0/12
		S	”	1	3	2	1	6	10.4	6/12
9	Mac	2	96*	0	0	0	0	5	0	0/5
		S	”*	0	1	2	0	2	8.0	3/5
10	De	2	120*	0	0	1	4	10	2.0	1/15
		5	144*	0	0	2	2	6	3.0	2/10
		0	—*	0	3	3	1	7	6.8	6/14

* Animals sacrificed 11 days after inoculation instead of at 10 days.

TABLE III.
Results of Treating Cotton Rats with Chloromycetin.

Exp. No.	Chloromycetin dosage		No. of animals with pulmonary lesions of				Avg L.S.	Infection ratio
	mg	Started at, hr	1	½	±	0		
3a	5	24	0	3	1	3	5.0	3/7
	S	”	3	1	1	1	10.8	4/6
4a	5	48	2	5	0	1	10.8	7/8
	20	”	3	1	0	2	11.6	4/6
	0	—	3	4	0	0	14.3	7/7
5a	20*	24	1	0	3	3	4.4	1/7
	S	”	1	1	1	4	5.0	2/7

* Given as divided doses of 10 mg each on first 3 days.

rat daily was given at various times up to 120 hours after inoculation. The results are presented in Table II. In Experiments 6 through 9 it is evident that an increase of the interval up to 96 hours did not significantly reduce the effectiveness of the antibiotic in preventing or eliminating the pulmonary lesions. In Experiment 10 the aureomycin was given at a dose of 2.0 mg daily starting at 5 days and of 5.0 mg daily starting at 6 days after infection. In this experiment the antibiotic appeared to be somewhat less effective than when started earlier. Small lesions were found in the lungs of some of the treated animals. In two instances, however, these lesions appeared to be much greyer than usual indicating that regression had begun earlier as a result of the treatment.

Results with Chloromycetin in cotton rats. Experiments with chloromycetin were done with daily doses of 5 and 20 mg starting treatment 24 to 48 hours after intranasal inoculation of the virus. The results are presented in Table III. In Experiment 3a the incidence of infection in the treated group receiving 5.0 mg of chloromycetin per day was similar to that of the control group but the lesions in the treated animals were on the average smaller than in the controls. In Experiment 4a the lesion scores and incidence of infection in the 2 treated groups were slightly lower than the controls. In the last experiment of Table III the dose of virus was so small that only 2 of the 7 controls developed pulmonary lesions, but one of the treated animals had rather extensive consolidation and in the lungs of 3 other animals there were minute spots and diffuse hyperemia graded as ± lesions.

TABLE IV.
Inhibitory Effect of Aureomycin on Growth of Atypical Pneumonia Virus in Chick Embryos.

Exp. No.	Atypical pneumonia strain	Dosage, mg/egg and time interval	Treated eggs		Control eggs	
			No. in pool	Result of passage to C.R.*	No. in pool	Result of passage to C.R.*
11	De	0.5 mg mixed with virus†	14	0/4	17	4/5
12	”	1 mg 2 hr after virus	12	0/6	15	4/7
13	”	1 mg 2 hr after virus	11	2/5	10	2/5
14	Mac	0.8 mg 2 hr after virus	4	2/8	7	5/8
15	”	1 mg 2 hr after virus	13	4/9	11	6/6
16	De	1 mg 2x (2 and 48 hr)	7	1/11	8	8/11
17	”	1 mg 2x (2 and 48 hr)	13	0/9	10	8/9
18	Mac	0.6 mg 2x (2 and 48 hr)	18	0/7	8	4/6

* Numerator represents number of cotton rats with lung lesions, denominator number tested.

† In this one experiment aureomycin at a concentration of 10 mg/ml and an equal part of virus suspension were mixed and 0.1 ml of the mixture inoculated into amnion.

Examination of the peritoneal cavity at autopsy of cotton rats receiving chloromycetin indicated good absorption of the antibiotic since only a few small particles remained.

Results with Aureomycin in Chick Embryos. Experiments in chick embryos were done to show that aureomycin actually inhibited multiplication of this virus because the objection could be raised that the results in cotton rats might be due to inhibition of the pathological reaction in the lungs. Such experiments in chick embryos also eliminated the possibility that the observed therapeutic effect was on infection with a latent virus carried by the animals themselves. In the first experiment aureomycin was mixed at a concentration of 10 mg per ml with 20% virus suspension and 0.1 ml of the resulting mixture inoculated without further incubation into the amnion of chick embryos. Cotton rats inoculated by the intranasal route with this material developed pulmonary lesions indicating the presence of active virus in the inoculum. Tests for the presence of virus in the treated chick embryos by subinoculation to cotton rats as described in the section on materials and methods were, however, negative. Control embryos were positive for virus. These results are summarized in the first line of Table IV (Experiment 11). The results of 4 subsequent experiments in which a single dose of 0.8 to 1.0 mg of aureomycin was inoculated into the yolk 2 hours after the virus were somewhat irregular (Experiments 12, 13, 14 and 15).

More significant effects were obtained when 2 doses of aureomycin were given 2 and 48 hours after the virus. In the total of 27 cotton rats inoculated from infected chick embryos in Experiments 16, 17, and 18, only one animal developed lung consolidation while 20 of the 26 animals receiving material from the control chick embryos developed pulmonary lesions. Since the material from the entire group of 7 to 18 treated chick embryos in each experiment was pooled for the tests in cotton rats this result indicates that aureomycin produced a considerable reduction of the amount of virus in every chick embryo.

Discussion. From the results presented it appears that the daily dose of aureomycin necessary for a definite therapeutic effect on the virus of atypical pneumonia in cotton rats varies from 20 to 100 mg per kilo of body weight depending upon the time after infection at which treatment is started. This is in good agreement with the dosage reported by other workers(1-4) to be effective against the disease in human beings. The oral dosage in man was between 2 to 6 g per day or 30 to 85 mg/kg. The exact nature of the agent causing primary atypical pneumonia has not been established but filtration data(6) indicate that it may be a representative of a class of agents sensitive to aureomycin but somewhat smaller than viruses of the psittacosis-lymphogranuloma group which are also inhibited by this drug.(10). In some of the experiments in human volunteers the agent is reported to have passed a Seitz filter (11,12) indicating a still smaller particle size. Re-

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peated microscopic examination of the infected tissues of human beings and experimental animals by a number of investigators has failed to reveal the presence of rickettsiae or elementary bodies. Although the experiments with chloromycetin in cotton rats were of a preliminary nature they leave little doubt that at a dosage up to 400 mg/kg the effects of this antibiotic against the agent of primary atypical pneumonia are irregular. The results parallel to some extent other observations with these two antibiotics against viruses of the psittacosis-lymphogranuloma group. Against intracerebral or intranasal infections of mice with these agents aureomycin apparently shows a greater activity than chloromycetin.(10,13,14)

Summary. Aureomycin injected intraperitoneally starting 24 hours after intranasal infection of cotton rats with the virus of primary atypical pneumonia almost completely inhibits the development of pulmonary consolidation when given in daily doses of 1 mg, and is partly active at a dosage of 0.5 mg per day. Therapeutic activity with doses of 2.0 to 5.0 mg daily was also demonstrable when the beginning of treatment was delayed for 5 to 6 days.

Chloromycetin in doses of 5 to 20 mg per day had slight and irregular effects on the virus in cotton rats under the same experimental conditions.

Aureomycin inhibits the growth of the atypical pneumonia virus in chick embryos when injected into the yolk sac in two doses of 1 mg each 2 and 48 hours after amniotic inoculation of the virus.

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The Huggins Cancer Test on 700 Normal Persons. (17564)

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Huggins *et al.*(1) have demonstrated that blood serum from persons with cancer, when heated at 100°C for 30 minutes, coagulates less readily than does normal serum. This defect in serum from persons with cancer can be accurately detected by the use of an inhibitor to coagulation, sodium iodoacetate, at a pH of 7.4. To permit mathematical expression of this phenomenon, Huggins proposed the "iodoacetate index"; the micro-moles of iodoacetate required to inhibit coagulation of one milliliter of serum, divided by the total protein of the serum in grams per cent. In a group of 283 individuals; 100 normals, 88 cancer patients, and 95 patients with non-malignant pathology; 85 clinically active cancers had indices of less than 9.0, as did 16 patients with non-malignant pathology. All normals fell within the range of 9.2 to 12.6. Since it is equally important to know how often one may expect an index below 9.0 in healthy persons, we have determined the iodoacetate index of 700 apparently healthy men and women of various ages at a large Army Training Center.

In our survey, the method, reagents, and equipment to determine the iodoacetate index were exactly the same as those used by Huggins and his coworkers.(2) The total serum protein was determined by the falling drop method, using the Eimer and Amend falling

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1. Huggins, C. B., Miller, G. M., and Jensen, E. V., *Cancer Research*, 1949, v9, 117.

2. Huggins, C. B., Miller, G. M., and Jensen, E. V., *Simplified Procedure for Serum Coagulation Test*, University of Chicago, 24 May, 1949.

TABLE I.
Frequency of Occurrence of Various Iodoacetate Indices on 700 Normal Persons.

Iodoacetate index	No. of persons
9.3– 9.5	6
9.6–10.0	24
10.1–10.5	71
10.6–11.0	164
11.1–11.5	185
11.6–12.0	156
12.1–12.5	80
12.6–13.0	11
13.1–13.5	2
13.6–14.0	1
	700

drop apparatus to attain the serum's specific gravity(3) and Weech's formula for total protein conversion.(4)

Serum specimens were obtained from 700 apparently healthy men and women at an Army Training Center. The age range of the individuals studied was from 17 to 56 years. Seventy-five percent were below age 24. Twenty-five sera of cancer patients were obtained from other hospitals and used as controls in the performance of the thermal coagulation tests on the normal sera. Tests were run in batteries of 40, with from 2 to 5 positive sera per battery as controls.

Results. Of the 700 individuals tested, the iodoacetate index of all fell within the range of 9.3 to 13.7. (Table I). The mean value was 11.27. The median was 11.10. Neither age nor sex significantly influenced the iodoacetate index. In 6 instances, the original determination yielded values of less than 9.3, ranging from 7.78 to 9.08. However, repetition of these determinations yielded normal values in each case, the results ranging from 9.76 to 12.18, so that they have been excluded from Table I. Of the 25 sera from cancer patients used for controls, 6 gave values higher than 9.3, ranging from 9.38 to 11.96. The remaining 19 values ranged from 3.06 to 8.95. Only those sera yielding an iodoacetate index of less than 9.0 were used as control sera in the survey. Of the 6 patients within the control group presumed to have cancer and who gave values within the normal range, 9.38 to 11.96, one was later determined to have a benign gastric polyp, and one was diagnosed as lymphosarcoma but had been receiving extensive x-ray therapy. The results in the 4 remaining cases are not explained. Their respective diagnoses are leukemia, adenocarcinoma of the rectum, carcinoma of the mandible, and generalized metastatic carcinoma, primary site unknown. One patient thought to have cancer of the cervix uteri gave an iodoacetate index of 4.24; however, a diagnostic curettage did not reveal malignant cells.

Discussion. It would seem that the iodoacetate index is uniformly negative in normal individuals. The technic is relatively simple and within the scope of the laboratory technician qualified to use an analytical balance in the preparation of the necessary reagents. The thermal coagulation endpoint is striking and quite reproducible. All determinations yielding abnormal indices should be repeated. On 6 occasions in which the initial index was below 9.3, a subsequent determination on different serum from the same individual yielded results above 9.3. The reason for this is not clear. However, in these cases, it was not possible to repeat the test with the original sera, so that experimental error may not be eliminated. In no instance of multiple determination upon the same serum specimen have the results varied more than one tube. In general, our findings on the sera of cancer patients coincide with those of Huggins. Since the clinical material was not under our direct control, it has not been possible to ferret out the explanation for normal results in supposed cancer patients nor the positive result in the one patient suspected of having, but not proven to have, cancer of the cervix.

Summary. The iodoacetate index was determined on 700 apparently healthy men and women of various age groups and found to be greater than 9.3 in all instances except in six cases in which normal values were obtained on repetition of the test. Seventy-five percent of the persons tested were less than 24 years of age.

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Changes in Endochondral Ossification of the Tibia Accompanying Acute Pantothenic Acid Deficiency in Young Rats.*† (17565)

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Retardation of growth of the tibia and grave impairment of chondrogenesis and endochondral ossification have been reported for chronic riboflavin deficiency in the rat.(2) These findings have prompted an investigation of the skeletal changes occurring in another B vitamin deficiency in the rat, namely pantothenic acid. Previous investigators have noted cartilage and bone marrow "hypoplasia" in pantothenic acid deficient rats.(3,4) Inhibition of growth and endochondral ossification has been observed also in pantothenic acid deficient mice.(5) In the present study the histological changes in the tibia accompanying pantothenic acid deficiency in young rats are described. The changes occurring in the oral mucosa and the mandibular joint of these rats have been reported.(6,7)

Experimental. Normal litters of 6 young together with the lactating mother (rats of the Long-Evans strain) were placed on the pantothenic acid-deficient or control diets at birth. Mother and young were weighed every fifth day; the young were weaned on the 21st day and continued on the same diet. This procedure is in contrast to the usual method of placing rats on experimental diets on the day of weaning (usually day 21) but has been found to produce the most acute pantothenic acid deficiency.(8) Suckling rats whose mothers were placed on the pantothenic acid deficient diet on the day of their birth, were severely retarded in growth and survived only a short time, *i.e.* the average survival for 226 rats was 35.4 ± 1.3 days (range 5-109 days) and only 13% lived past 60 days of age. In the majority of the young death occurred between their 25th and 40th day of age. The deficiency was so acute that many of the animals died before 30 days of age severely retarded in growth but without developing any other readily observable symptoms of the deficiency. In the animals that survived past 45 days of age, the characteristic symptoms of the deficiency (porphyrin deposition, dermatitis and greying) developed in some of the animals.

The pantothenic acid deficient diet was composed of 24% alcohol-extracted casein, 64% sucrose, 4% McCollum salts No. 185(9) and 8% hydrogenated cottonseed oil (Crisco). To each kilogram of diet was added 2 mg thiamine, 2 mg pyridoxine, 5 mg riboflavin, 5

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† A preliminary report of this study was given at the 24th General Meeting of the International Association for Dental Research, March, 1946.(1)

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TABLE I.
Effect of Acute Pantothenic Acid Deficiency in Male Rats on Tibial Length and Epiphyseal Cartilage Width.

	Pantothenic acid control			Pantothenic acid deficient		
Age, days	Body wt at autopsy, g	Tibial length, mm	Epiphyseal cartilage width, mμ	Body wt at autopsy, g	Tibial length, mm	Epiphyseal cartilage width, mμ
26	58	25.8	410	20	16.9	243
34	56	25.8	410	28	19.2	213
45	79	28.9	376	35	23.5	152
61	140	33.5	355	40	24.1	152
97	260	37.2	116	46	25.4	87*
Avg	119	30.2	333	34	21.8	179

* This excludes 50 micra of calcified sealing-off bone.

mg p-aminobenzoic acid, 10 mg nicotinic acid, 200 mg inositol and 500 mg choline chloride. During lactation the mother of each litter, both deficient and control, received weekly 1 cc of a fat-soluble vitamin mixture containing 6 mg alpha-tocopherol, 115 chick units vitamin D, 800 U.S.P. units vitamin A and 650 mg corn oil (Mazola). After weaning each individual rat received 0.5 cc of this fat-soluble vitamin mixture weekly by stomach tube. Control animals received the identical purified diet supplemented with 28 mg calcium pantothenate per kilogram of diet; in a few cases 500 μg calcium pantothenate was given weekly from the day of weaning. In addition, young together with their mothers were maintained from birth on a diet of natural foods§ as normal controls.

For the histologic study, 20 pantothenic acid deficient young (13 male and 7 female rats) that were most severely affected as judged by retardation of body weight and external appearance were sacrificed at ages ranging from 3 to 16 weeks. These rats were usually moribund at the time of autopsy. Rats receiving the purified diet supplemented with calcium pantothenate and normal rats reared on a diet of natural foods were autopsied at the same time. The tibias were fixed in 10% neutral formol, roentgenographed and measured, decalcified in 5% nitric acid, embedded in nitrocellulose, sectioned at 8 micra, and stained with hematoxylin and eosin.

Results. The histologic aspect of the epiphyseal cartilage of the tibia in normal rats reared on a diet of natural foods and the changes occurring with increasing age have been published in detail.(10) In this study tibial lengths and epiphyseal cartilage widths of the pantothenic acid-supplemented animals maintained on the purified diet were slightly but consistently below normal at every age. Histologically, increased lipid deposition in the marrow and slight involvement of hematopoiesis were observed. Rats receiving calcium pantothenate only from weaning did not approach normality as closely as rats receiving the vitamin throughout their entire life. Similar findings with other purified diets have recently been reported in a study of chronic riboflavin deficiency in the rat.(2) Tibial measurement showed considerable differences between the pantothenic acid-deficient and supplemented animals (Table I). The average tibial length for the deficient group was 21.8 mm as contrasted with 30.2 mm for the vitamin-supplemented group. The width of the epiphyseal cartilage also reflected corresponding decreases, averaging 179 and 333 micra for the deficient and supplemented

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§ This diet is McCollum's Diet I, slightly modified, and contains 67.5% ground whole wheat, 15% technical casein, 10% whole milk powder, 5.25% hydrogenated vegetable oil (Crisco or Primex), 1.5% calcium carbonate, and 0.75% iodized sodium chloride, 3.5 g Sardilene (fish oil concentrate containing 3,000 U.S.P. units vitamin A and 400 Chick units vitamin D per g) are added to each kilogram of diet.

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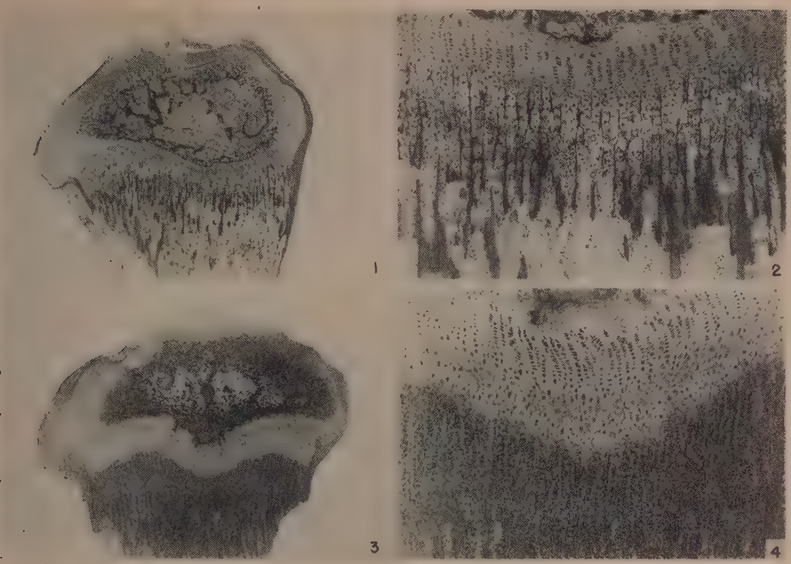

PLATE I.
Proximal epiphysis of the tibia of male rats. Hematoxylin and eosin stain.
FIG. 1. Pantothenic acid deficient, 26 days of age, plate 9680, × 30.
FIG. 2. Pantothenic acid deficient, 26 days of age, plate 9696, × 140.
FIG. 3. Control, 26 days of age, plate 9703, × 30.
FIG. 4. Control, 26 days of age, plate 9706, × 140.

groups, respectively. The histologic changes observed in the tibia pantothenic acid-deficient rats fall into four distinct classes which do not necessarily correlate with age. Due to variations in individual response to the deficiency, increasing severity of morphologic disturbance has been used as the sole criterion for classification.

STAGE I: This stage was found in rats at 21, 26 and 33 days of age and is best represented by the tibia of the 26-day-old rat (Figures 1 and 2). Although the epiphysis is small and possesses thick articular cartilage, much early calcification is seen along the inner margin. The epiphyseal trabeculae are thin and scant. The epiphyseal cartilage is one-half the width of the corresponding vitamin-supplemented control, the decrease occurring almost entirely in the basophilic layer. The columns are slightly less regular than normal and are separated by increased amounts of matrix. Capillary tufts are directed against the cartilage cells of the vesicular zone and erosion appears active. The diaphyseal trabeculae are short, coarsened and are seldom covered with osteoblasts. However, osteoclasts possessing 8-10 nuclei are profuse. Along the endosteum the proliferation of osteoblasts is retarded and osteoblasts undergoing lysis may be seen. The diaphyseal medulla is granulocytopenic and erythrocytopenic and contains no fat cells. The marrow is highly edematous and characterized by many large connective tissue cells. In addition, cells somewhat similar to macrophages and connective tissue cells but larger and containing much clear substance without macrophagic inclusions are seen. The tibia of the 26-day-old rat supplemented with calcium pantothenate is shown in Fig. 3 and 4 and represents a typical picture for the age range of 21-36 days. The epiphysis is larger than that just described and many fine trabeculae are directed centrally. The metaphysis is wide and composed of long, straight columns of basophilic cells separated by little matrix. Erosion is proceeding rapidly and the trabeculae are slender and long with little anasto-

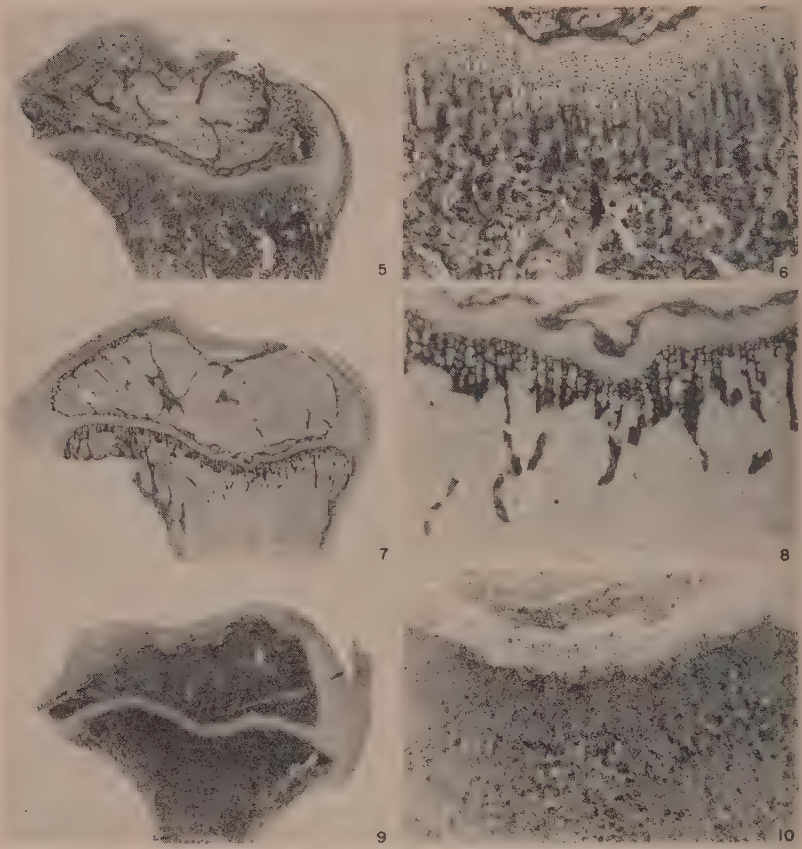

PLATE II.
Proximal epiphysis of the tibia of male rats. Hematoxylin and eosin stain.
FIG. 5. Pantothenic acid deficient, 61 days of age, plate 9978, × 30.
FIG. 6. Pantothenic acid deficient, 61 days of age, plate 9924, × 140.
FIG. 7. Pantothenic acid deficient, 45 days of age, plate 9683, × 30.
FIG. 8. Pantothenic acid deficient, 45 days of age, plate 9697, × 140.
FIG. 9. Pantothenic acid deficient, 97 days of age, plate 9981, × 30.
FIG. 10. Pantothenic acid deficient, 97 days of age, plate 9704, × 140.

mosing. Occasionally osteoclasts are encountered subjacent to the epiphyseal cartilage. Osteoblasts are present in abundance at the capillary tips and along the trabeculae and the endosteum. The diaphyseal marrow is slightly granulocytopenic and fat cells may be noted in the marrow cavity.

STAGE II: This stage is observed over an age range of 33 to 63 days and is represented by the 61-day-old tibia (Fig. 5 and 6). The cartilage is less than half the width of the corresponding vitamin-supplemented control. Erosion is decreased. The trabeculae are blunt and thick and osteoclasts are present. The osteoblasts at the remaining capillary tufts are so small and fibroblast-like as to merit comment and osteoblastic proliferation along the endosteum has ceased. Small pointed fibroblasts frequently act as replacements. In the marrow varying degrees of granulocytopenia or erythrocytopenia are encountered. In the vitamin-supplemented controls for this early age range (not illustrated), the larger epiphyses, wide epiphyseal cartilage, numerous long trabeculae and the profusion of osteoblasts contrast with the marked retardation of endochondral ossification in the deficient rats.

STAGE III: In this stage the deficiency has now resulted in the virtual cessation of growth. Calcification has sealed off the inner margin of the epiphysis and the diaphyseal side of the epiphyseal cartilage. Chosen from an age range of 45 through 72 days, the 45-day-old animal exemplifies this condition (Fig. 7 and 8). The fragile appearance of the epiphysis with its marked absence of trabeculae as the result of continued resorption is striking. The osteoblasts remaining at the juxtadiaphyseal region are small; fibroblasts line some of the few trabeculae and no endosteal proliferation is evident. The absence of erythrocytes is pronounced in the 45 and 48-day-old tibias. Otherwise the granulocytopenia previously noted in the marrow persists.

STAGE IV: This stage represents the final arrest of growth. All tibias for rats between 78 and 109 days reflected similar histologic pictures and the 97-day-old tibia may serve as an illustration for this period (Fig. 9 and 10). The epiphysis has not increased in size and persists in its frailty and absence of trabeculae. Bone growth is impaired by sealing-off bone of approximately 50 micra in the seven animals investigated. The epiphyseal cartilage is narrow and shows much matrix in the basophilic zone. Only a few very small osteoblasts and osteoclasts remain. No fat storage is seen in the marrow cavities. Hematopoiesis in some rats was improved with regard to erythrocytes but not to granulocytes. Occasional heavy basophilic staining of the marrow is due to the high incidence of blast forms, premyelocytes and metamyelocytes and the paucity of older forms. The 78-109 day old rats supplemented with calcium pantothenate showed a larger epiphysis with many heavy ramifying trabeculae. The epiphyseal cartilage was progressively reduced with age(10) but lacked the sealing-off bone observed in the deficient group. Through this age range the vitamin-supplemented rats showed diminished osteogenic activity whereas in the deficient group complete cessation of osteogenesis had occurred as early as 78 days.

Discussion. The data reported in this study demonstrate that pantothenic acid is a vital factor for the stimulation and maintenance of normal chondrogenesis, osteogenesis and hematopoiesis in the rat. Histologic study of the tibia has revealed severe disturbance to morphologic structure depending more upon individual response to the deficiency than upon the age of the animal. In all stages of the deficiency growth of the tibia was retarded; chondrogenesis and endochondral ossification were drastically reduced. Hematopoiesis was usually severely affected, the bone marrow being granulocytopenic and/or erythrocytopenic and lacking megakarocytes. In Stages III and IV the onset of calcified sealing-off bone along the inner margin of the epiphysis and the diaphyseal margin of the epiphyseal cartilage showed that growth of the tibia had stopped. In normal rats of the Long-Evans strain the beginning of sealing-off bone in the tibia has been shown to occur at approximately 170 days of age(10) but complete sealing-off never occurs.

The inhibition of chondrogenesis and endochondral ossification observed in this study is much more severe than that previously reported for pantothenic acid deficient rats (or mice). This is to be expected in view of the acute syndrome produced by instituting the deficiency much earlier in life. Sealing-off bone has not been observed previously in pantothenic acid deficient animals although its formation at a comparable early age has been reported for rats deficient in vitamin A(11) and for hypophysectomized rats.(12)‖ The marked impairment of hematopoiesis observed in this study is in agreement with the findings of Daft *et alia*(4) on pantothenic acid deficient rats. These investigators also reported a variable degree of edema in the bone marrow in contrast to the markedly edematous condition found in the youngest animals of this study. A similar markedly edematous bone marrow has been reported for rats de-

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‖ In this laboratory sealing-off bone at this early age has also been observed in acute protein deficiency in the rat (to be published).

ficient in pteroylglutamic acid(13) and "serous atrophy" of the bone marrow, possibly a comparable condition, for rats restricted in calories(14) and for mice depleted of the entire vitamin B complex.(15) The inhibition of chondrogenesis and endochondral ossification previously observed in chronic riboflavin deficiency in the rat(2) is similar to that reported here for acute pantothenic acid deficiency. In comparison, the acute pantothenic acid deficiency was characterized by marked resorptive activity which led to the complete absence of diaphyseal trabeculae and the formation of sealing-off bone at a much earlier age. In addition, fatty infiltration of the bone marrow, which was marked in riboflavin deficiency, was not observed in this acute pantothenic acid deficiency.

Despite the severity of the morphologic changes produced by the lack of pantothenic acid and the prevention of practically all changes by the administration of the synthetic vitamin, it is not to be inferred that all of these changes are due specifically to the lack of this vitamin. Biotin and pteroylglutamic acid were not present in the purified diets used in this study nor in the diets used by other investigators carrying out pathological studies of pantothenic acid-deficient animals. Daft *et alia*(4) have presented evidence indicating that the granulocytopenia occurring in pantothenic acid-deficient rats is due to an induced deficiency of pteroylglutamic acid. These authors also found evidence indicating that the anemia observed in such animals might be due either to pantothenic acid deficiency or to a deficiency of an unknown vitamin. The effects of pteroylglutamic acid deficiency and caloric restriction have been mentioned in connection with the edematous bone marrow. Caloric restriction, as well as pantothenic acid deficiency, also induces a pteroylglutamic acid deficiency(16) and it is probable that some of the effects previously attributed to caloric restriction may actually be due to deficiencies of pteroylglutamic acid or of still unknown vitamins. The role of caloric restriction, pteroylglutamic acid and of unknown factors in the changes in endochondral ossification accompanying acute pantothenic acid deficiency in young rats obviously needs further investigation.

Summary. The changes in endochondral ossification of the tibia accompanying acute pantothenic acid deficiency in young rats have been investigated. Twenty deficient animals, 13 male and 7 female rats, were autopsied together with their controls at ages ranging from 3 to 16 weeks. The histologic changes observed were decreased growth of the tibia with marked impairment in chondrogenesis, osteogenesis and hematopoiesis.

Four stages of morphologic changes were observed. Stage I, in which the ages of the rats ranged from 21 to 33 days, was characterized by unusual trabecular resorption, decreased osteoblast proliferation and edema of the bone marrow. In Stage II the ages ranged from 33 to 63 days and marked retardation of osteogenesis with some calcification in the epiphysis and blunt diaphyseal trabeculae was observed. The dominant feature of Stage III, from 45 to 72 days, was the beginning of epiphyseal cartilage calcification and the cessation of osteogenesis. Stage IV, from 78 to 109 days, was characterized by complete absence of trabeculae and the formation of a heavy layer of sealing-off bone below the epiphyseal cartilage.

The role of pantothenic acid and of additional factors such as pteroylglutamic acid, unknown vitamins and caloric restriction in these histologic changes is discussed.

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Effect of Choline as a Lipotropic Agent in the Treatment of Human Coronary Atherosclerosis.*† (17566)

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Evidence is continually accumulating that lipotropic agents such as choline, a member of the vitamn B complex, are effective in the prevention of experimental and clinical fatty infiltration and degeneration of the liver,(1,2) and in experimentally induced atherosclerosis.(3-4) Numerous observers(4-7) have described evidence from which it was concluded that a disorder in lipoid or cholesterol metabolism is a significant factor in the development of human atherosclerosis. In view of the encouraging lipotropic action of choline on atherosclerosis in experimental animals, the authors inaugurated a study of the lipotropic action of choline in proven human atherosclerosis over a 3-year period.

Method of Study. A group of 230 patients were studied who were admitted in consecutive order to the Los Angeles County General Hospital medical wards and were proven to have acute coronary thrombosis with myocardial infarction, as demonstrated by typical serial electrocardiograms, history, laboratory tests and physical findings. These patients were thereupon accepted as proven cases of coronary atherosclerosis. One hundred and fifteen of the patients served as "controls"; these were discharged from the hospital on recovery after an average of 6 weeks, and were then followed over a 3-year time period. They were not given choline; some were given whatever medication may have been indicated for symptoms; in cases that were symptom-free no medication was prescribed but the patients' progress was followed periodically. The other group of 115 patients represented those who were admitted in alternate order to the Los Angeles County General Hospital for their first acute myocardial infarction. After they were discharged from the hospital they were placed on choline treatment over a 3-year period, and followed in the Research Clinic of the Hospital. Fifty-two patients were given choline for 1 year, 35 patients took choline for 2 years, and 28 patients were given choline for 3 years. The dose of choline varied from 6 to 32 g daily per individual depending on their tolerance for the drug and the degree of hypercholesterolemia present. Choline bicarbonate was used in this study. The diet remained the same as that taken by the patients prior to their infarction; patients with their first known infarction only were studied. The ages for both controls and choline treated cases ranged from 30 to 70 years, and from 28 to 70 years respectively; most patients were of the white race and were males.

Results. Of the 115 control patients 35 patients or 30% had died after 3 years. Death in this series was due similarly either to recurrent coronary thrombosis with myocardial infarction (19 cases), congestive heart failure (10 cases), or extracardiac causes (6 cases). In the choline-treated series of 115 patients, 14 patients or 12% had died after 3 years.

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† Appreciation is hereby expressed to Dr. Lawrence W. Smith, for the supply of choline bicarbonate, and to Albert L. Chaney, Lillian Hall, and Perla Berlin, for their assistance.

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Death in this series was due similarly either to recurrent coronary thrombosis with myocardial infarction (6 cases), congestive heart (5 cases), or to extracardiac causes (3 cases). Upon comparison the mortality rate mainly from cardiac cause in the control series was 30% as against 12% for the choline treated patients.

Discussion. Previous clinical and experimental observations on the action of choline in preventing or mitigating experimental atherosclerosis have suggested that this lipotropic agent like that of its fellow members of the vitamin B complex inositol,(5) pyridoxine,(8) and methionine,(5) appears to prevent arterial atheromatous deposition or to exert a decholesterolizing effect on the atheromatous deposits in the vascular walls of experimental animals,(3,4,5) and in man.(6)

Summary. Over a 3-year period the lipotropic agent choline was effective in significantly reducing the mortality rate due to recurrent coronary thrombosis with myocardial infarction in a series of 115 patients with proven coronary atherosclerosis.

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Portal Blood in Collateral Veins of Patients with Cirrhosis. Acetylation by the Intestine. (17567)

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Few direct studies on absorption from the gastro-intestinal tract have been carried out in humans because of the inaccessibility of the portal vein. The use of the London angiostomy technique(1) for obtaining portal vein blood shed considerable light on the absorption process in animals, but due to the difficulty of the procedure the studies were limited. Furthermore, the applicability of these studies to humans is uncertain. Sherlock and Walsh(2) made the interesting observation that a large superficial collateral vein over the abdomen of a patient with cirrhosis of the liver could be shown to contain portal blood. Their observations were restricted, however, because the vessel became thrombosed after the injection of radio-opaque dye. Three other patients with cirrhosis failed to show evidence of portal blood in their abdominal veins. The present investigation was undertaken to ascertain the incidence of superficial abdominal veins carrying portal blood in patients with cirrhosis of the liver and to devise a simple method for detecting such channels. A large proportion of patients with cirrhosis were found to show abdominal portal collaterals. Two simple techniques for their detection are described, together with preliminary physiological studies using the proven portal collaterals. After this work was completed, Billings and DePree(3) reported observations on glucose absorption in abdominal veins of patients with cirrhosis. They also found a high incidence of portal collaterals.

The dynamics of the absorption process as revealed in portal (abdominal collateral vein) and peripheral (antecubital vein) blood are illustrated in Fig. 1. A patient with cirrhosis and ascites was given 2.5 g of sulfadiazine orally and the concentration of the total drug in the blood was determined by the method of Bratton and Marshall.(4) The level in the

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* Public Health Service Research Fellow of the National Institutes of Health.

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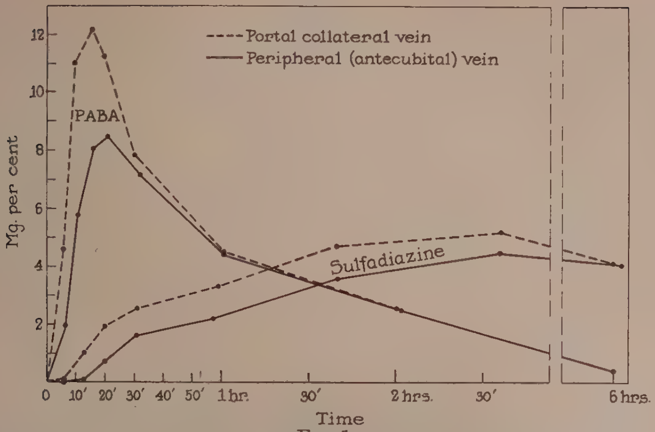

FIG. 1.
Portal (collateral vein) and peripheral (antecubital vein) blood levels after feeding 2.5 g of sulfadiazine and 2.5 g of PABA on separate days.

portal blood rose to 1 mg% before any significant amount of drug appeared in the peripheral blood. The level was consistently higher in the portal blood until after the peak of absorption, when the levels in the two systems became equal. The same patient on another occasion was fed 2.5 g of p-aminobenzoic acid. Because of the more rapid absorption of this drug, there were wider differences between the levels in portal and peripheral blood until the peak of absorption was reached. Following this, the level in the portal blood began to fall, while that in the peripheral blood was still rising. Both levels then dropped to reach the same concentration, and remaining equal, fell further as the drug was eliminated rapidly from the blood stream.

Since the determination of sulfonamides is tedious and time consuming, substances that could be measured more easily were used to demonstrate portal blood. Fig. 2A shows the levels of thiocyanate in the peripheral and portal blood after an oral dose of 1 g of sodium thiocyanate. Thiocyanate is rapidly and simply determined by the method of Crandall and Anderson(5) modified for use of only 1 cc of serum. The determination of fluorescein is even simpler and requires only a fraction of a cc of blood. After feeding 0.25 g of sodium fluorescein (Fig. 2B), 0.1 cc of serum was diluted in 10 cc of weak base and the level read directly in the photofluorometer. In the fasting state thiocyanate and fluorescein are almost completely absorbed within 45 to 60 minutes. Fifteen minutes after ingestion the level in the portal blood is approximately twice that in the peripheral blood. An additional determination at 25-30 minutes may be used to confirm the wide difference in levels diagnostic of the portal

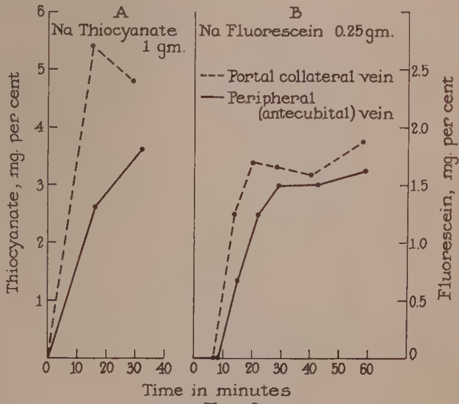

FIG. 2.
Portal (collateral vein) and peripheral (antecubital vein) blood levels after feeding of thiocyanate and fluorescein in different patients.

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TABLE I.
Acetylated and "Free" PABA Levels in Portal and Peripheral Blood after Oral Administration.

Dose in tablets	Time of samples, min.		Acetyl-PABA, mg %		"Free" PABA, mg %	
	Portal	Peripheral	Portal	Peripheral	Portal	Peripheral
0.5 g PABA	16.5	21	0	0	0	0
	23.5	26.5	.05	0	0	0
	32	37	.09	.05	.07	0
	47	49.5	.14	.08	.09	0
	82	84.5	.20	.17	.06	0
	119	125.5	.21	.23	.15	0
	164	169	.28	.25	.29	.06
2.5 g PABA	7.5	9	0	0	0	0
	12.5	14	.22	0	0	0
	21.5	24	.29	.17	0	0
	72.5	74	.37	.37	1.23	.62

origin of abdomnal vein blood. Glucose and other substances with high initial levels may also be used to demonstrate portal blood. They require much larger oral doses than do foreign substances to raise the blood levels significantly.

These rapid procedures for the diagnosis of portal collateral channels were applied to 12 patients with proven esophageal varices and other signs of portal hypertension resulting from cirrhosis of the liver. Of 8 patients with ascites, portal collaterals were demonstrated in 6. Two of these patients showed portal blood in more than one collateral vein. Abdominal veins in the region of the epigastrium tended to be the most likely sources of portal blood. Of 4 patients without well marked ascites, only one gave a positive test. The level of the test materials in the patients classified as negative was either the same or lower in the abdominal veins than in the antecubital veins. The reason for negative results in cases with proven portal hypertension has not been determined. While significantly higher levels of test drug in an abdominal than in an antecubital vein definitely indicate portal origin, identical or lower values apparently do not disprove portal origin.

All substances absorbed through the portal system that were tested were found in higher concentration in the abdominal than in the peripheral veins of these patients. These include sodium, sulfapyridine, glucose, and amino acids, as well as the various drugs discussed above. Evidence was obtained that blood from widely separated areas of the portal bed may drain into the same collateral vein. After PABA was given by mouth and thiocyanate by rectum, both drugs were found in higher concentrations in the abdominal vein tested than in peripheral blood.

In the course of studying PABA absorption evidence for acetylation by the intestine was found. PABA was fed as compressed sugar coated tablets, so that absorption was slowed. Table I shows the results in 2 patients in whom portal blood contained only acetylated PABA, without any "free"† PABA, at a time when neither compound had yet entered the peripheral circulation. The acetylation was apparently accomplished by the intestinal tract. After the first appearance of acetylated PABA in the portal system, subsequent samplings showed higher portal than peripheral levels. This phenomenon of higher portal than peripheral levels of acetyl-PABA was demonstrated again in both of these patients on repeating the experiment; it also occurred in 2 other patients tested. When PABA was absorbed slowly, the portal blood contained chiefly acetylated PABA and very little of the "free" drug was present and available for acetylation by the liver. However, in other experiments where PABA was fed as a rapidly absorbed solution, a large amount of "free" PABA appeared in the portal blood which was then acetylated principally by the liver. As a result of this evidence it appears that under the circumstances where small amounts of material are slowly absorbed, intestinal acetylation assumes relative importance.

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† "Free" refers to unacetylated PABA; it includes a fraction conjugated with other radicals.

Summary. 1. Portal origin of blood in abdominal collateral veins of patients with portal hypertension may be demonstrated simply and rapidly by feeding fluorescein or thiocyanate and finding higher concentrations in the abdominal veins than in the antecubital veins.

2. Seven out of 12 patients with cirrhosis gave positive tests for the portal origin of abdominal collateral vein blood. Patients with acites are more likely to give positive results than those without ascites.

3. Evidence is presented for the acetylation of PABA by the human intestinal tract.

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Effect of Inhibitors on Phosphate Uptake in Excised Gills of the Mussel (*Mytilus edulis*). (17568)

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The factors limiting the rate of penetration of phosphate into the ciliated epithelium constituting the molluscan branchial tissue have been discussed.(1) The present study is a survey of the effects of some commonly-used metabolic inhibitors (cyanide, azide, sulfide, fluoride, iodoacetate, and cysteine) upon the phosphate uptake process.

Materials and methods. Excised fragments of the gills of *Mytilus edulis* L., a pelecypod mollusk, were obtained as previously described and placed in a shallow glass irrigation cell equipped with a thin cover glass.(1) Immediately above this was a counting tube of a Geiger-Müller circuit. The tissue was irrigated with an artificial sea water (ASW) containing 5 micromols of phosphate per liter, of which a minute fraction was labelled with radioactive phosphorus (P^{32}). The radioactivity of the tissue was estimated at intervals of about 10 minutes. When sufficient information had been obtained to describe the uptake of the isotope under these conditions, the flow of irrigant was stopped, and a new fluid was introduced containing, in addition to the other components mentioned, an inhibiting agent in 0.001 M concentration. Readings were then continued until sufficient information was obtained pertaining to the new conditions. At the conclusion of observations the tissue was removed from the glass cell and examined with the compound microscope to observe the ciliary activity. All experiments were performed at 15°C.

Results. Phosphate content of the gill fragment was expressed as a percentage of the tissue radioactivity at the time of addition of the inhibitor. Fig. 1 shows the results of plotting the data so adjusted. The heavy broken lines connect the averages obtained with each inhibitor. The lighter smooth curves, identical in each graph, show the expected phosphate uptake, based on 22 previous experiments,(1) in which the value of k, the phosphate saturation constant, was found to be 0.0050 min.$^{-1}$ The administration of cyanide, sulfide, or iodoacetate resulted in a phosphate uptake less than expected, while addition of azide or fluoride was without pronounced effect. An apparent stimulating effect of cysteine is shown in the last curve.

Rigorous statistical treatment of the data was impractical for several reasons, but an idea of the significance of the observed differences may be obtained by comparing the relative value of radioactivity obtained at a

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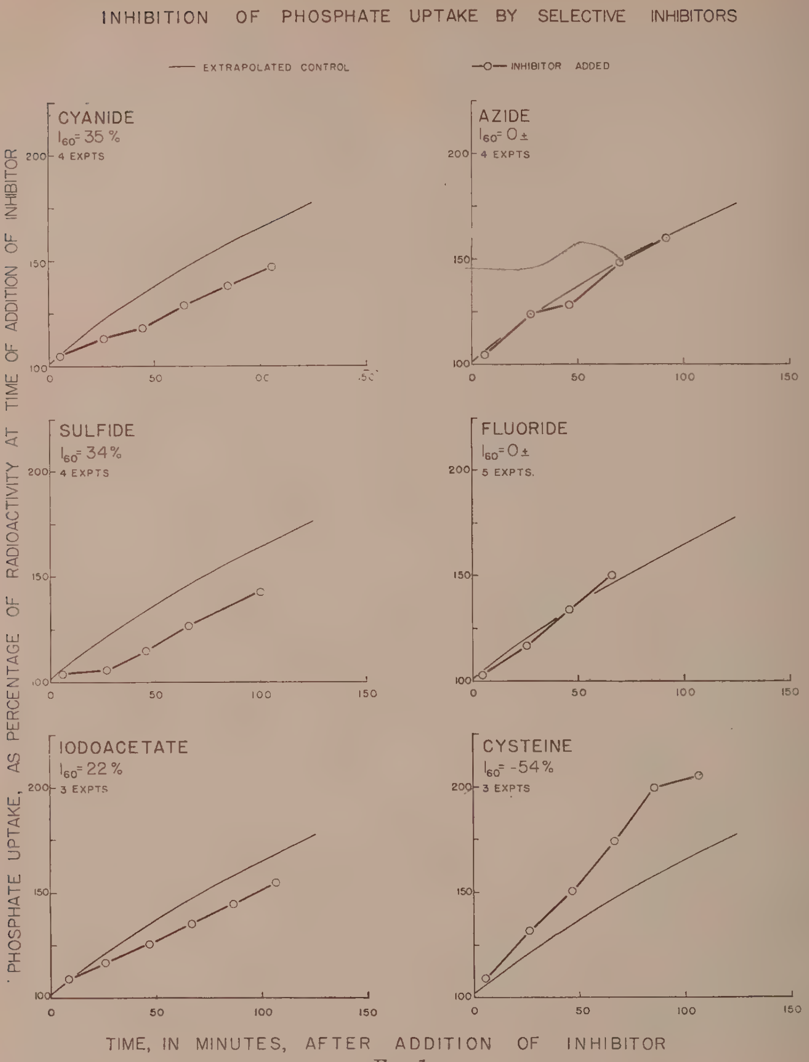

FIG. 1.

specified time after the addition of the inhibiting agent, with that at the corresponding time on the control curve. Such comparisons ["Student's" t-test(2)] were made for the observations at 60 minutes after addition of the inhibitor; the probabilities of the differences being associated with chance variation are as follows: cyanide, 0.001; sulfide, 0.001; iodoacetate, 0.05; azide, 0.5; fluoride, 0.6; cysteine, 0.014. The results of these comparisons confirm the impression that the inhibitions observed with cyanide, sulfide, and possibly iodoacetate are real effects, while the differences observed with azide and fluoride could easily arise as the result of sampling error. Microscopic examination of the gill after each experiment revealed no perceptible slowing of the ciliary beat even after 2 hours

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TABLE I.
% of Each Inhibitor Remaining Undissociated at pH 8.0 (the pH of ASW).

Inhibitor	Dissociation	Constant, as pK	Author	% undissociated
Cyanide	$HR = H^+ + R^-$	9.14	(6)	93
Azide	$HR = H^+ + R^-$	4.72	(6)	.05
Sulfide	$H_2R = H^+ + HR^-$	7.04	(6)	10
”	$HR^- = H^+ + R^{--}$	14.92	(6)	100
Fluoride	$HR = H^+ + R^-$	3.16	(7)	.001
Iodoacetate	$HR = H^+ + R^-$	3.15	(7)	.001
Cysteine	$RCOOH = H^+ + RCOO^-$	10.28	(4)	95
”	$RNH_2 + H_2O = RNH_3^+ + OH^-$	12.04	(4)	100
”	$RSH = H^+ + RS^-$	8.18	(4)	60

immersion in the inhibitor.

Discussion. Two loci at which these reagents may affect the uptake of phosphate must be considered: a) the cell surface, affecting the permeability of the membrane to phosphate; and b) the cell interior, affecting the rate of turnover of organically bound phosphate, probably through selective inhibition.

In general, substances having the properties of weak acids penetrate only in the undissociated form.(3) The degree of dissociation depends on the pH and the ionic strength of the medium in which they are dissolved. Table I gives the proportion of each inhibitor in the undissociated form, using the dissociation constants available from the literature, and calculated from them by means of a suitable form of the Henderson-Hasselbalch equation.(4) Since no allowance was made for the difference between the ionic strength of ASW and that of distilled water, these figures are approximate. The table shows that cyanide, sulfide (as HS^-), and possibly cysteine, considered from this point of view, would penetrate readily and would be able to act within the cell. Iodoacetate, azide, and fluoride would penetrate scarcely at all, existing as they do almost entirely in the ionic state. Fluoride is known to penetrate cells in the ionized form, but only very slowly compared with chloride and bromide ions.(3)

Results of the present study indicate that the inhibition of phosphate uptake may take place at both loci mentioned above. Iodoacetate is unlikely to penetrate the cell rapidly; any inhibition by this reagent would suggest a general effect on the proteins at the surface of the cell. The ubiquity of glycolysis, and the observed lack of inhibition by fluoride and azide tend to confirm the nonpenetration of these strong electrolytes, and strengthen the case for possible surface action by iodoacetate.

Cyanide and sulfide, both able to penetrate the cell, were effective inhibitors. Note, however, that these affect enzyme systems which are also known to be sensitive to azide, which itself was ineffective. It is therefore suggested that cyanide and sulfide exert their inhibitory action by affecting metabolic turnover within the cell. No explanation is offered for the effect of cysteine, in the presence of which the uptake rate rose slowly. Though many systems are activated in the presence of cysteine,(5) these are usually of the proteolytic sort; it is difficult to correlate the scanty material available.

Summary. Excised fragments of gills of *Mytilus edulis* L., a pelecypod mollusk, were irrigated with artificial sea water containing P^{32}. The rate of uptake of phosphate in the presence of 0.001 M inhibitors was compared with the control rate. Inhibition of phosphate uptake in the presence of cyanide, sulfide, and iodoacetate, and the lack of inhibition by azide

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and fluoride, are considered from the point of view of available knowledge of the penetration of such substances into cells. Cyanide and sulfide, being salts of weak acids, penetrate the cells and probably affect the turnover rate of intracellular phosphate. The lack of effect with fluoride is consistent with its existence in the completely dissociated state. Iodoacetate, also completely ionized, may be effective at the cell surface, affecting the permeability of the cell to phosphate. The apparent rise in the rate of phosphate uptake in the presence of cysteine remains unexplained.

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The Sulfhydryl Content of Normal and Abnormal Human Sera.* (17569)

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The important role of the sulfhydryl group in metabolic processes has long been recognized although the precise manner in which it participates in these reactions is not clearly understood.(1,2) Kolthoff and Harris(3) have described an amperometric method for the determination of mercaptans and this technic has been adapted as a quantitative method for sulfhydryl groups in amino acids and proteins by Benesch and Benesch.(4) During the past 3 years, we have been conducting studies on the mechanism of action of various drugs and one of the determinations which has been of great interest has been that of the sulfhydryl content of the serum proteins. It was hoped that an indicator system could be developed which would aid not only in clarifying the metabolic abnormalities induced by the presence of a neoplasm but also measure the effect of chemotherapeutic agents. Although the objective of this study was to follow the progressive changes which occur in animals and patients with tumors, it also seemed desirable to investigate patients with other diseases in which changes in the serum proteins are known to occur.

Methods. Blood was obtained from patients in the fasting state and the serum carefully separated to avoid hemolysis. The fresh serum was then added to 21% methanol solution containing ammonium nitrate and ammonium hydroxide as supporting electrolytes. The current which flows between a mercury-mercuric iodide electrode and a rotating platinum electrode through the serum-methanol solution is measured with a sensitive galvanometer. As .001 N silver nitrate is added in small increments, a silver mercaptide is formed and the increment of current is small. When all the free SH groups have reacted, a slight excess of silver ion results in a relatively large increase in the diffusion current. By plotting graphically, the current flow observed against the added silver nitrate, one obtains two straight line curves whose intersection is the equivalence point.(3) The titrations are standardized with cysteine each day and the results are expressed as milligrams of cysteine per unit, *i.e.*, per 100 ml serum, gram protein nitrogen, gram albumin nitrogen, etc. It is not implied however, that the sulfhydryl determined in serum is cysteine. Proteins have been separated into albumin and globulin components by the method of Pillemer,(5) and the SH determined in each individually. Protein

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4. Benesch, R., and Benesch, R. E., *Arch. Biochem.*, 1948, v19, 35.

5. Pillemer, L., and Hutchinson, M. C., *J. Biol. Chem.*, 1945, v158, 299.

TABLE I.
Summary of Sulfhydryl Determination in Human Sera.

Status of patients	No. of determinations	SH* per 100 ml serum	Total N† g per 100 ml	SH* per g N
Normal				
Male	9	6.53 ± .24	1.20 ± .06	5.46 ± .27
Female	10	6.36 ± .25	1.19 ± .03	5.36 ± .30
Abnormal				
Cancer	46	4.28 ± .23	1.13 ± .14	3.84 ± .48
Infection	13	5.03 ± .63	1.22 ± .12	4.17 ± .61
Degenerative disease‡	12	4.92 ± .95	1.25 ± .08	3.85 ± .74
Miscellaneous states§	12	2.70 ± .91	1.06 ± .15	2.76 ± .97
Leukemia	34	4.56 ± .65	—	—

* Expressed as mg of cysteine. Values are given as the mean ± one standard deviation (S.D.). When less than 20 observations are recorded, the S.D. has been multiplied by $\sqrt{\frac{n}{n-1}}$.

† No correction was made for serum N.P.N.

‡ Includes asthma, arteriosclerosis, hypertension, arthritis, etc.

§ Includes cirrhosis of liver, lupus erythematosus disseminata, chronic nephritis and hemachromatosis, in which reversal of the albumin-globulin ratio was present.

content was determined by a micro-Kjeldahl method and also by the color developed by a quantitative biuret reaction. The details of these methods will be presented at a later date.

Results. The values presented in the table show a distinct difference in the serum sulfhydryl content of normal individuals when compared with sera obtained from patients who were suffering from various disease states. The latter are lower in all cases. It should be noted that the mean deviations recorded are those of biological individuality and can not be attributed to technic. Duplicate sulfhydryl determinations on an individual serum showed less than a 2% variation while the nitrogen values agreed within one percent. The total serum protein as measured by nitrogen and biuret determinations revealed no evidence of hypoproteinemia in any of the groups. It is evident, as presented in the last column of the table, that the sulfhydryl determined per gram of nitrogen did not differ significantly from one another among the abnormal sera.

Discussion. The results presented indicate that clinical abnormality can be detected by reduction in the serum sulfhydryl content, although our experience with this determination, on whole sera, does not permit utilization of this method for specific diagnosis as *e.g.*, the presence or absence of cancer. Studies in progress have revealed that 80 percent of the total normal serum sulfhydryl is in the albumin fraction. A reduction in the amount of albumin might be expected to result in lower sulfhydryl values per unit volume of serum. This *quantitative* factor does contribute to the lower sulfhydryl of abnormal sera. A *qualitative* factor, as yet unexplained, is also present, in that the sulfhydryl per gram of albumin nitrogen is below normal. The same qualitative change occurs in the globulin fraction despite the quantitative increase of globulin which often occurs. These findings indicate an altered protein synthesis in certain diseases which results in fewer sulfhydryl groups available for this titration.

The data of Brand(6) show 4 mols of cysteine per mol of human albumin and 9 mols per mol of gamma globulin. The values we have found for normal serum albumin average 0.68 mol available cysteine per mol of albumin. Hughes(7) has reported the isolation of a crystalline mercury salt of human albumin, from which the mercury could be removed by dialysis against 0.001 M cysteine. His recovery would correspond to 0.66 mol of cysteine per mol of total albumin. Thus it appears that we are titrating the same groups in human serum albumin. Desoxyribonucleic acid, cre-

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6. Brand, E., *Ann. N. Y. Acad. Sci.*, 1946, v47, 187.

7. Hughes, W. L., Jr., *J. Am. Chem. Soc.*, 1947, v69, 1836.

atine, or formaldehyde when added to serum did not affect the sulfhydryl value previously determined.

The enzymes which are associated with anabolic processes, the cathepsins, are known to be activated by sulhydryl compounds. Altered protein synthesis and sulfhydryl content may be related to such intracellular enzyme systems. Huggins, *et al.*,(8) have studied changes in thermal coagulability, in the presence of iodoacetate, as a measure of serum protein alterations in disease. Iodoacetate is a well known reagent for sulhydryl groups. The iodoacetate indices determined by Huggins, *et al.*, for normal and abnormal sera are of the same order of magnitude as the sulfhydryl content determined with the amperometric method. Although we do not consider the determination of serum sulfhydryl to be a specific diagnostic test for cancer, it may serve as an additional objective indicator in following animals and patients receiving chemotherapy.

Summary. The sulfhydryl content of serum proteins has been quantitatively determined through an adaptation of an amperometric titration. Among normal adults, the values noted, expressed as milligrams of cysteine, were uniform, reproducible and averaged 6.5 ± 0.2 mg per 100 ml serum or 5.5±0.3 mg per gram serum nitrogen. The albumin component of serum contained 80% of the total sulfhydryl. The addition of desoxyribonucleic acid, creatine or formaldehyde did not change the sulfhydryl content of the serum. The addition of an SH reagent such as para-chloro-mercuri-benzoate abolished all reactant groups measured with this method.

Sera obtained from patients with various diseases showed a significant reduction in the sulfhydryl available for this titration. This quantitative reduction in sulfhydryl was still evident when corrected for differences in the albumin and globulin components and thus represented a qualitative change in the serum proteins. At the present time, the alteration in the sulfhydryl content of serum or its components is not characteristic enough to permit specific differentiation among the neoplastic, metabolic, or infectious diseases. The possible application of serial serum sulfhydryl determinations in animals and patients may be of value as an objective indicator of response and for study of the mechanism of action of chemotherapeutic and other agents.

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8. Huggins, C., Miller, G. M., and Jensen, E. V., *Cancer Res.*, 1949, v9, 177.

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Surface Fixation. A New Method of Detecting Certain Immunological Reactions. (17570)

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The study of rapid agglutination tests for the diagnosis of a number of infections has shown that carefully prepared and standardized antigens are of considerable help to the practitioner as well as the epidemiologist. The use of a suspension of formalinized and blue-stained Proteus X-19 in slide agglutination tests with whole blood(1) has been a satisfactory preliminary method for the diagnosis of typhus fever. This encouraged us to apply the same method to brucellosis. We have had excellent results. Both tests are performed on a slide. A 3 mm wire loop of antigen is mixed with whole blood taken with a 2 mm loop. The mixture is rotated in order to favor the margination of the agglutinated antigen. In about 1 or 2 minutes a thick blue ring forms and surrounds the red cells which have separated from the antigen. In negative reactions the mixture remains a uniform greenish color often surrounded by a reddish ring formed by marginated red cells. The forma-

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1. Castaneda, M. R., Silva, R., and Monnier, A., *Rev. Med. del Hospital General*, 1940, v8, 382.

tion of rings also occurs when the test is performed in a concave slide or in a white porcelain plate. This phenomenon can be of great usefulness when applied to the identification of closely related antigens such as *Br. melitensis* and *Br. abortus.* These organisms are stained in different colors; *i.e.,* blue and red. If the purple mixture is treated with a proper amount of anti-abortus or anti-melitensis serum and rotated a red or blue ring becomes apparent at the edge of the drop. The test which we described under the name of selective agglutination(2) seems to be the consequence of a very rapid union of homologous antigen and antibody leading to the clumping and margination of the particles which adhere to the porcelain wall. The formation of these rings either in blood tests or in selective agglutination is unexplained by the usual theories of orthodox agglutination tests. The work of Northrop(3), Boyd(4) and others, however, leads to a rather simple explanation. If a bacterial suspension is considered as a lyophobic colloid, which loses its zeta potential under the influence of an antibody coating, the agglutinated particles are readily pushed away from the menstruum to become attached to the edge of the drop. While studying this type of serological reaction we made an observation which, if previously known, has not been mentioned in the available medical literature. Since it may have theoretical and practical importance we wish to describe the finding. Its simplicity makes us rather dubious that others have not noticed it.

Preparation of Antigen and Test. The antigens used for this test are prepared as follows: *Brucella abortus* and *Br. melitensis* are cultivated during 48 hours in liver infusion agar. The growth is washed with saline and filtered through cotton. Formalin is added to a concentration of 10%. The suspension is left at room temperature for 24 hours, then centrifuged and the sediment resuspended in a small volume of distilled water. This suspension is treated with ferrous sulphate as recommended by Huddleson and Carrillo(5) for the preparation of milk antigens. The suspension is stained with a fresh aqueous solution of hematoxylin (Coleman and Bell) and becomes nearly black. The stained suspension is left over night, centrifuged and resuspended in 4 volumes of isotonic NaCl solution.

The filter paper used in this experiment was E. D. No. 609. The test may be performed by the two following methods:

(a) The serum and antigen are mixed on a slide by means of drops carried with a wire loop. One loopful of the mixture is transferred to the lower end of a strip of filter paper about 1 cm from the edge. As soon as possible, to avoid evaporation, stand the strip of paper in a layer of isotonic NaCl solution 0.5 cm deep in a manner similar to the procedures used in paper chromatography. The flow of fluid will go rapidly over the mixture which may or may not be washed upward with the ascending current of saline.

(b) The phenomenon may be observed placing a wire loopful of 4 mm of serum on a piece of filter paper. When the serum has reached its maximum spread, a drop of antigen taken with a 2 mm loop is deposited carefully in the center of the serum-spot. With the same loop drops of isotonic saline are placed over the antigen and may or may not push the antigen from the place where it was deposited.

Results. In procedure (a) if isotonic NaCl is used on the spot on the filter paper, the current of saline may wash upward part of the black antigen (Fig. 3), but most of it remains where it is on the strip just as occurs when diluted Parker No. 51 black ink is used instead of antigen (Fig. 1). If normal serum is used in the mixture most of the antigen is washed upwards with the ascending fluid leaving little or no trace of it at the bottom of the paper (Fig. 4). This occurs also when we use the ink instead of antigen (Fig. 2). When the mixture is prepared with antigen

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2. Castaneda, M. R., *J. Immunology,* 1942, v43, 203.

3. Northrop, J. H., *The Newer Knowledge of Bacteriology and Immunology,* pp. 782-801, Jordan and Folk, The Chicago University Press, 1929.

4. Boyd, W. C., *Fundamentals of Immunology,* Interscience Publishers, Inc., N.Y., 1943.

5. Huddleson, I. F., and Carrillo, C. D., *Veterinary Medicine,* Chicago, 1949, v64, No. 6.

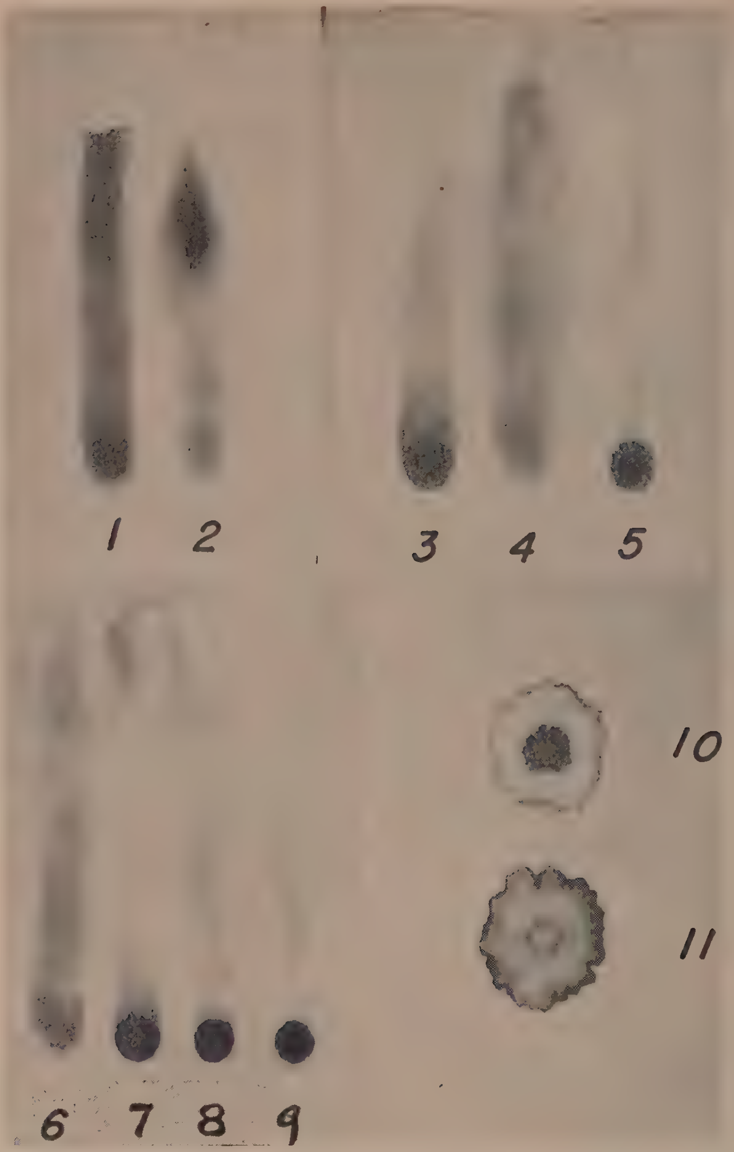

FIG. 1. Mixture of diluted Parker No. 51 ink with isotonic saline solution. Notice that the ink, in spite of being washed upwards in part, remains largely at the bottom of the paper.

FIG. 2. Same ink mixed with human serum. Little or no ink left at the bottom.

FIG. 3. Brucella antigen in a mixture with isotonic saline solution. Remains largely at the bottom.

FIG. 4. Same antigen in a mixture with "normal" human serum. Notice that it is washed upwards.

FIG. 5. Immune serum and antigen mixture. Becomes attached to the spot where it was placed.

FIG. 6 to 9. This shows various intensities of the reaction starting with a negative in 6 to a strong serum in 9.

FIG. 10. Spot test using serum from a case of brucellosis. Notice that fixation of the antigen has occurred in the center of the spot. FIG. 11. Spot test with a negative serum.

and serum from a person known to have brucellosis in evolution, the immune serum antigen mixture leaves a black spot practically unaltered at the place where it was set. A control mixture containing normal serum is washed upwards. The different behavior of both mixtures is very striking and takes place in less than 1 minute (Fig. 5).

The intensity of the test varies in degree as shown in Figs. 7, 8, and 9. Fig. 6 is a normal serum control.

The main difference in the action of normal and immune serum seems to depend on the fact that normal serum acts as a protective colloid upon the antigen (as it acts upon the ink), preventing its fixation in the paper from which it may be readily washed away by the ascending fluid. When immune serum is used in the test the protective effect fails to act upon a "colloid" previously modified in its electrical condition because of the attachment of antibody. Under such conditions the reaction becomes similar to that of a mixture of saline and antigen though considerably reinforced by the action of the specific globulin.

Because of this affinity to the filter paper by the immune serum-antigen mixtures, we believe that the designation of "surface fixation" may be explanatory of this phenomenon.

The union of the antigen and antibody seems to be very rapid which is to be expected from the findings on agglutination and precipitation reactions by the authors cited herewith and a very demonstrative addition to this view may be observed using method (b). When the antigen is placed in a previously arranged wheal of normal serum the addition of saline will push the antigen to the periphery producing a more or less uniform spread of stained organisms (Fig. 11). This occurs in a fraction of a second. If the antigen is set on a spot of immune serum, the addition of saline is without noticeable effect upon the antigen which remains at the place where it was placed (Fig. 10).

The intensity of the fixation phenomenon depends on the antibody content of the serum. It is demonstrable with serum from patients suffering from brucellosis in evolution and remains active even when agglutinins have become of very low titres. The application of this method as a test for doubtful cases of brucellosis has not been investigated *in extenso.* So far, only antigens prepared with Brucella have been studied. All organisms do not have the same ability to spread with the ascending fluid, which shows certain relationships to phenomena studied in paper chromatography.

Summary. A new method of demonstrating the union of antigen and antibody is described. The test is performed on the surface of filter paper and submitted to the washing effect of a stream of isotonic NaCl solution. While normal serum is washed together with the antigen, the immune serum produces the fixation of the antigen to the paper at the place where it was deposited.

Regarding its specificity, it has been observed that this test, studied only in brucellosis, produces responses which so far have agreed with the usual agglutination reactions.

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Disappearance of Hyaluronidase from the Blood of Albino Rats. (17571)

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Following the intravenous injection of hyaluronidase into the albino rat, edema of the extremities, elevation of the blood hematocrit and a decrease of the concentration of circulating plasma proteins result.(1,2) These effects are most marked in 15-45 minutes and

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1. Elster, S. K., Freeman, M. E., and Dorfman, A., *Am. J. Physiology*, 1949, v156, 429.

2. Elster, S. K., Freeman, M. E., and Anderson, P., *J. Lab. and Clin. Med.*, 1949, v34, 834.

are no longer observed in 4-8 hours. The present investigation was undertaken to study the rate of disappearance of the intravenously administered hyaluronidase and the changes produced by it in the level of the nonspecific plasma hyaluronidase inhibitors.

Experimental: One hundred twenty-four male albino rats of the Sherman strain, weighing between 140 and 270 g were fed Purina Laboratory Chow and water *ad lib.* The hyaluronidase was prepared from fresh bull testes according to the method of Freeman *et al.*(3)* The material was assayed and measured 3,500 turbidity reducing units (T.R.U.) per mg of nitrogen. These units have been defined by the assay procedure of Dorfman and Ott.(4) Seventy-four animals were anesthetized with ether and the jugular vein was exposed. Seven thousand five hundred T.R.U. hyaluronidase in normal saline were injected intravenously. At 5, 15 and 30 minutes and 1, 2, 4, 8, 24 and 72 hours, groups of animals were bled from the aorta with a heparinized syringe. The blood was centrifuged and the plasma separated. The hyaluronidase content of these plasma samples was determined turbidimetrically. The plasma hyaluronidase inhibitor level(5) of these and of 13 normal male albino rats was measured. Since the methods of assay measure the excess of one over the other, the values for hyaluronidase and plasma inhibitor are relative.

Thirty-seven rats were placed in metabolism cages and their urines collected for a period of 18 hours. The animals were then injected intravenously with 7,500 T.R.U. hyaluronidase, and they were replaced in the metabolism cages. Urine specimens for the subsequent 18 hours were collected in containers kept at 0-5°C. The hyaluronidase content of 15 and the protein content of the remaining 22 samples were determined.(6)

TABLE I.
Plasma Hyaluronidase Level Following Intravenous Administration of 7500 T.R.U.

Time following inj.	No. of animals	Mean plasma level (T.R.U./cc)	Range (T.R.U./cc)
5 min.	11	604	369–1000
15 ”	9	65.3	47–110
30 ”	7	4.4	0–16
1-4 hr	10	0	0

TABLE II.
Plasma Inhibitor Level Following Intravenous Administration of 7500 T.R.U. Hyaluronidase.

Hr following inj.	No. of animals	Mean plasma level (μ/cc)
1	6	<40
2	6	<40
4	10	<40
8	5	46.6
24	5	69.0
72	5	72.2

Mean normal plasma inhibitor level (13 rats)—75.1 μ/cc.

Following the introduction of testicular hyaluronidase into the blood, its plasma concentration was maximal at 5 minutes (Table I). It rapidly diminished, and at 30 minutes 5 of 7 specimens had amounts insufficient for detection; of the remaining 2, one contained 15 and the other 16 units/cc.

In Table II are listed the changes of the plasma hyaluronidase inhibitor following the intravenous injection of the enzyme. The mean value of sera of 13 normal rats was 75.1 units/cc. Since hyaluronidase was present in the 5, 15 and 30 minute samples, the inhibitor level could not be measured. At 1, 2 and 4 hours the amount of hyaluronidase inhibitor was less than 40 units/cc; at 8 hours it was still diminished, and it returned to normal levels in 24 to 72 hours.

Very small amounts of hyaluronidase appeared in the urine of the rats following the administration of 7,500 T.R.U. The mean control urine volume of the 15 rats for the 18 hours preceding the injection was 8.5 cc. The mean urine volume for the same time period following injection was 7.1 cc. The average hyaluronidase content of the urine

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3. Freeman, M. E., Anderson, P., Oberg, M., and Dorfman, A., *J. B. C.*, 1949, v180, 655.

* The authors are indebted to Major Monroe E. Freeman, MSC, for the hyaluronidase preparations.

4. Dorfman, A., and Ott, M. L., *J. B. C.*, 1948, v172, 367.

5. Dorfman, A., Ott, M. L., and Whitney, R., *J. B. C.*, 1948, v174, 621.

6. Simmons, J. S., and Gentzkow, C. F. Laboratory Methods of the United States Army, Lea and Febiger, 1944, Philadelphia.

was 22.3 T.R.U./cc, so that a total of 158 T.R.U. hyaluronidase was excreted. There was no increase of the urinary protein excretion of 22 rats. During the control 18 hour period, 5.7 cc of urine were excreted, containing 1.33 mg protein per cc, or a total of 7.6 mg protein. During the comparable post-injection interval, the animals passed 5.5 cc of urine, that contained 1.33 mg protein per cc for a total of 7.3 mg protein.

Discussion: Meyer(7) has written that "intravenously injected enzyme is rapidly eliminated from the circulation". Seifter(8) found in rabbits a short sojourn of the injected hyaluronidase in the blood as assayed with the mucin-clot method. It was considerably longer when measured by the spreading technique. In the rabbit and the dog, large amounts of the hyaluronidase were excreted in the urine. In the albino rat, intravenously administered hyaluronidase disappeared rapidly from the blood. Approximately 2% of the enzyme was found in the urine in an active turbidity reducing form. The amount of hyaluronidase injected into the animal represented 12.5 mg protein. The absence of significant proteinuria was added evidence of the lack of renal excretion of the enzyme. The fate of the injected enzyme has not been elucidated completely. Hyaluronidase most probably is a small protein molecule(9) and may be lost by simple diffusion from the vascular bed. This diffusion may be increased by the specific action of the enzyme *in vivo*, in which fluid and proteins are actively passed through the capillary wall.(1,2) A small amount of the enzyme was lost in the urine, and an unknown amount was inactivated by the blood inhibitors. As yet, the remainder has not been traced.

The plasma inhibitors were diminished by the administration of hyaluronidase, suggesting a direct action of one on the other. Recovery to normal levels did not occur for 24-72 hours. The source of the newly formed inhibitors has not been found. Wattenberg and Glick(10) have suggested that the inhibitors may be formed in the blood stream itself, since the tissues of the body had no anti-hyaluronidase activity.

Summary: Following the intravenous administration of testicular hyaluronidase to albino rats, the following changes were noted:

1. The maximum concentration of the enzyme was attained within 5 minutes and diminished rapidly, so that at one hour it was no longer detected by turbidimetric methods.
2. Approximately 2% of the hyaluronidase appeared in the urine in an active form.
3. The plasma hyaluronidase inhibitor level was depressed for 8 hours and returned to normal within 24-72 hours.
4. No significant proteinuria resulted.

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8. Seifter, J., *Ann. N. Y. Acad. Sci.*, in press.

9. Freeman, M. E., personal communication.

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Destructive Action of Astatine^{211} (Element 85) on the Thyroid Gland of the Rat.* (17572)

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The observation that astatine accumulates in the thyroid suggested that this new element might be of possible therapeutic application in hyperthyroidism as has already been demonstrated in the case of radioiodine.(1,2) As-

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tatine is not only radioactive with a half-life of 7.5 hours,(3) but unlike most of the artificial radio-elements, it emits alpha particles† rather than electrons and gamma rays. The radiobiological significance of the fact that astatine^{211} emits alpha particles is that each of these nuclear particles arising from the disintegration of an astatine nucleus will dissipate an initial kinetic energy of 6 Mev in about 50 μ of a soft tissue such as the thyroid. The beta rays from the 8-day radioiodine, I^{131}, possess a maximum energy of .6 Mev with a range of nearly 2,000 μ of tissue.

Methods. The At^{211} was produced by the bombardment of bismuth by 29 Mev alpha particles from the 60 inch cyclotron at the Crocker Laboratory. The astatine^{211} was distilled from the bismuth target and collected in 12 N HCl, which in turn was diluted and made isotonic by the addition of an appropriate quantity of sodium hydroxide. The procedures outlined above were derived from a report by Segre and his co-workers which describes the nuclear and chemical properties of astatine.(4) Preliminary thyroid uptake studies in the rat revealed that in a group of eight 200 g animals of both sexes that from 2% to 8% of the administered astatine^{211} was present in the thyroid at the end of 18 hours,(5) the necessary corrections for the decay of the astatine^{211} having been applied. These values are comparable to the results obtained in earlier studies in which guinea pigs were employed as the experimental animals.(1) More detailed studies on the distribution of astatine^{211} in other tissues are now in progress. Three 200 g white rats were selected to observe the radiobiological action of the alpha particle irradiation of the thyroid from the accumulated astatine^{211}. The astatine^{211} in an isotonic solution of NaCl was administered to two of the rats by intraperitoneal injection. They received 10 μc and 50 μc respectively. The third animal was used as a control. After 30 days the 3 animals

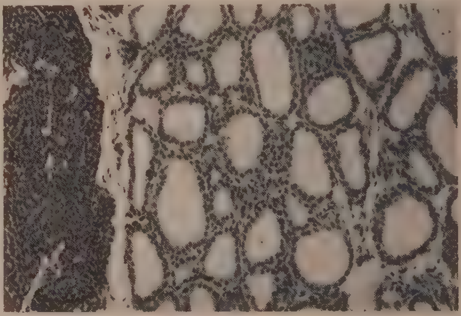

FIG. 1.
Photomicrographs of thyroid and parathyroid tissue of rats. H&E, × 140.
Normal control. (Bouin's fixation).

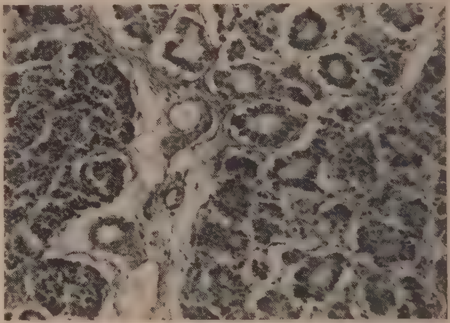

FIG. 2.
Thirty days after injection of 10 μc of At^{211}. (Alcohol fixation).

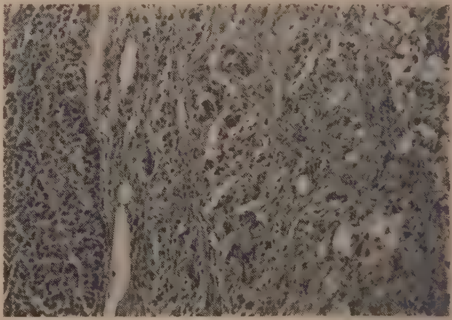

FIG. 3.
Thirty days after injection of 50 μc of At^{211}. (Alcohol fixation).

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† In addition there are emitted x-rays of 80 Kev which makes possible *in vivo* thyroid uptake studies in man.(2)

4. Johnson, G. L., Leininger, R. F., and Segre, E., *J. Chem. Phys.*, 1949, v17, 1.

5. Unpublished data.

were sacrificed and the thyroids removed. The tissue was fixed, embedded in paraffin, and the sections stained with haematoxylin and eosin for histological study. The sections were cut in such a manner that from each of the 3 thyroids a portion of the parathyroid gland was included.

Results. Photomicrographs of the sections from the control, 10 μc, and 50 μc studies are shown in Fig. 1, 2 and 3 respectively. At the 10 microcurie dosage level it is apparent that there was a marked degree of injury to the thyroid follicles as evidenced by their diminished size, loss of colloid, and increase in size of the follicular epithelium, Fig. 2. The general thyroid architecture is distorted to such a degree that it would seem probable that in this instance the degree of radiation injury approached a level of being totally irreversible even if a recovery period of from six months to a year had been allowed. The adjacent parathyroid tissue did not reveal any observable changes that would suggest radiation injury to this organ. The thyroid tissue in the rat which received 50 microcuries of astatine^{211} is not recognizable as such. Here there is complete obliteration of the follicles, colloid, and follicular epithelium. The thyroid tissue has been replaced by fibrous tissue with some infiltration of lymphocytes and fat cells. In this instance it appears that a complete and irreversible destruction of the thyroid gland took place, as a result of an extreme degree of radiation injury. It is significant to note that there was no histological evidence of manifest radiation injury in the adjoining parathyroid tissue.

Summary. The results from this preliminary study demonstrate the capacity of astatine to induce an extreme degree of radiation injury of the thyroid gland in the rat without apparent involvement of the adjoining parathyroid gland.

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Prevention of Procaine Convulsions by Presidon and Sodium Pentobarbital. (17573)

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While the use of procaine is extensive and relatively safe, an occasional subject is found sensitive to the drug. The toxic reactions to subcutaneous procaine are caused by stimulation of the central nervous system resulting in restlessness and tremors which may proceed to convulsions. There is also an initial period of respiratory stimulation followed by depression of the medullary centers resulting in dyspnea and respiratory collapse. The use of hypnotics in the prevention and treatment of toxic reactions to local anesthetics is based on animal studies which show that hypnotics, especially the barbiturates, depress the convulsive effects of local anesthetics.(1,2) Presidon (Pyrithyldione; 3,3-diethyl-2,4-dioxotetrahydropyridine) is a relatively short acting hypnotic with a wide margin of safety.(3,4) It was shown to inhibit the convulsions produced by caffeine,(3) picrotoxin and Metrazol.(4) It was found to be an effective hypnotic in human subjects.(5) We have extended the studies on the anticonvulsive properties of Presidon to the prevention of pro-

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3. Krautwald, A., Kuschinsky, G., and Riedel, H., *Arch. Exp. Path. Pharmacol.*, 1939, v193, 219.

4. Koppanyi, T., Herwick, R. P., Linegar, C. R., and Foster, R. H. K., *Arch. intern. pharmacodynamie*, 1940, v64, 123.

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caine convulsions in various species. The comparative hypnotic and anticonvulsive properties of Presidon and pentobarbital have been determined in mice, guinea pigs, rabbits and dogs. Similar studies in rats were abandoned because of the high resistance of the rat to procaine intoxication.

Methods. The convulsive dose of procaine administered subcutaneously was determined for each species. The toxic symptoms included excitability, nervousness, tachycardia, dyspnea, clonic convulsions and terminal respiratory failure. In addition, rabbits and guinea pigs displayed opisthotonos and tonic extension of the limbs; tonic extension was common to the dogs as well. The dose which produced these toxic effects in 95% of the animals was calculated from a log dose-probit graph and the standard errors also calculated.(6) The hypnotic dose for pentobarbital and Presidon administered intraperitoneally was determined as the dose which produced sleep in 50% of the animals. The endpoint of hypnosis was the loss of righting reflexes *i.e.* the animal was unable to right itself when placed on its side. The hypnotic doses and standard errors were calculated by the probit method.(6) The protective doses of pentobarbital and Presidon were determined as the doses which prevented convulsions in 50% of the animals which had received the 95% convulsive dose of procaine. The hypnotic drugs were given intraperitoneally 10 minutes prior to the subcutaneous administration of procaine. The endpoint was the prevention of the convulsive movements in the various species. The protective doses were calculated by the graph probit method.(6)

In order to compare the relative effectiveness of pentobarbital and Presidon, protective indexes were calculated as the ratio of the hypnotic dose to the protective dose.

Results. The results summarized in the table show that Presidon in comparison with pentobarbital administered intraperitoneally is an effective anticonvulsant. The protective index for Presidon is higher than for pentobarbital in the four species studied. The dose

TABLE I.
Hypnotic and Anti-convulsant Effects of Presidon and Pentobarbital.

Treatment		Mice, mg/kg	Guinea pigs, mg/kg	Rabbits, mg/kg	Dogs, mg/kg
Procaine HCl	Convulsive dose—CD95 ± s.e.	1000 ± 250	140 ± 8.0	225 ± 20.3	410 ± 62
Pentobarbital sodium	Hypnotic ,, —HD50 ± s.e.	38 ± 3.2	6.5 ± 1.2	11.3 ± 1.2	5.1 ± 5.0
Presidon	,, ,, —HD50 ± s.e.	178 ± 3.6	87 ± 4.4	87 ± 12.2	35 ± 14
Pentobarbital sodium	Protective ,, —PD50 ± s.e.	40 ± 12.0	2.95 ± 1.6	5.5 ± 2.1	2.9 ± 0.6
Presidon	,, ,, —PD50 ± s.e.	42 ± 18.5	28 ± 14.6	22.5 ± 14.8	13 ± 1.5
Pentobarbital sodium	,, index—$\frac{HD50}{PD50}$	.95	2.2	2.06	1.75
Presidon	,, ,, —$\frac{HD50}{PD50}$	4.2	3.1	3.7	2.70

Procaine was administered subcutaneously; pentobarbital and Presidon were administered intraperitoneally.

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of Presidon which protects mice from procaine convulsions is about one-fourth the hypnotic dose but the protective and hypnotic doses of pentobarbital are almost identical. In the other species the protective dose of Presidon is about one-third the hypnotic dose whereas the protective dose of pentobarbital is about one-half the hypnotic dose. There is large species variation in the sensitivity to procaine convulsions and also to the hypnotic and anticonvulsive potency of pentobarbital and Presidon. The mouse is most resistant to both procaine and the hypnotics. The dog is more resistant than the guinea pig or rabbit to procaine convulsions but is most sensitive to the hynotics. The smallest protective doses of the hypnotics were required by the dog.

Discussion. In cases of procaine intoxication, there is an initial period of respiratory stimulation which is followed by depression which may lead to terminal respiratory failure(2,7,8). Most deaths following the subcutaneous administration of procaine were caused by respiratory failure even though, in some instances, the convulsions had been controlled.(9,10) In view of the strong depressant action of the barbiturates upon respiration, it would seem more feasible to employ a prophylactic drug which could abort the convulsive effects of procaine HCl without causing additive effects on the already depressed respiration. Presidon and pentobarbital have been compared as central nervous system depressants using hypnotic activity as an index. That Presidon has a wider margin of safety than representative barbiturates has been previously reported.(3,4) It is important to note that pentobarbital confers protection against the convulsive effects of procaine HCl only in doses which are considerably more depressant than equally protective doses of Presidon. It would seem, therefore, that the ability of a drug to prevent local anesthetic intoxication does not parallel its degree of depressant action on the central nervous system.

Summary. Presidon and pentobarbital administered intraperitoneally, prevent the convulsions produced by procaine in dogs, rabbits, guinea pigs, and mice.

Presidon confers protection against the convulsive effects of procaine at one-fourth to one-third the hypnotic dose, whereas the protective dose of pentobarbital is from one-half to the full hypnotic dose.

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Relationship of Type of Growth of *M. tuberculosis* to Antituberculous Activity of Subtilin.*† (17574)

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In the course of an investigation of the antimicrobial activity of subtilin it was observed that although this substance possessed a high degree of inhibitory activity on tubercle bacilli *in vitro*, it failed to afford protection to mice experimentally infected with tuberculosis,

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TABLE I.
Increase in Activity of Subtilin on Tubercle Bacilli Associated with the Diffuse Growth in the Presence of Tween 80.

Concentration of Tween, %	Min. inhibitory conc. of subtilin, μg/ml	Type of growth in control
.0	>100	Clumped
.001	>100	,,
.005	50	,,
.01	3.12	Partially clumped
.02	3.12	Diffuse
.05	3.12	,,
.1	1.56	,,
.2	1.56	,,
.4	1.56	,,
.8	1.56	,,

despite prolonged administration of relatively large doses. These findings were in accord with those of other investigators, Anderson and Wong,(1) and Wong, Hambly, and Anderson,(2) who had noted similar activity of subtilin *in vitro,* but failure to modify the course of experimental tuberculosis in hamsters. There are numerous examples of drugs which exhibit antibacterial activity *in vitro* but lack therapeutic value. Nevertheless, many of the recognized causes of loss of antibacterial activity *in vivo* appeared to be excluded in the case of subtilin by virtue of the demonstration in several laboratories, including our own, that subtilin was highly active *in vivo* on other bacteria such as pneumococci and streptococci.(3-5) Further study, however, disclosed that the activity of subtilin *in vitro* was maintained only in the presence of a suitable detergent, such as Tween 80,§ which was contained in the culture medium employed in the tests mentioned above. Fischer(6) has reported that certain strains of tubercle bacilli recovered from patients receiving streptomycin may be inhibited by low concentrations of that drug in a medium containing Tween, but are resistant to as much as 1000 μg per ml in other culture media. Enhancement of the action of penicillin on tubercle bacilli by Tween has been reported by Kirby and Dubos,(7) who suggested the possibility that the increase in activity of penicillin might be correlated with the degree of growth dispersion of the bacilli. The findings with subtilin suggested that this factor might also be operative in determining the degree of activity exerted by this drug on tubercle bacilli.

Accordingly, an investigation was made of the effect of the type of growth of tubercle bacilli on the antituberculous activity of subtilin, under various conditions which modify the degree of dispersion of the microorganisms.

Materials and methods. The *in vitro* tests were carried out in the Tween-albumin medium described by Dubos and Davis,(8) which was modified in certain experiments by variations in the concentration of Tween, or by the addition of serum. The test organism employed was *M. tuberculosis* (H37Rv), and the results reported are those recorded after incubation for seven days. Further incubation was continued, but caused no significant differences in the results. Bioassays of subtilin in serum were performed with a conventional serial dilution technic using as test organism *Streptococcus hemolyticus* (C203 MV) and *Staphylococcus aureus* (Oxford H

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2. Wong, S. C., Hambly, A. S., and Anderson, H. H., *J. Lab. and Clin. Med.,* 1947, v32, 837.

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§ Tween 80 (sorbitan monooleate), is a product of the Atlas Powder Co., Wilmington, Del.

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strain). The subtilin used in this study was obtained from two sources|| and the results with all samples were in substantial agreement.

Results. The first group of tests was devised to determine the activity of subtilin on tubercle bacilli under conditions in which the type of growth was varied by cultivation in graded concentrations of Tween 80. The inhibitory concentrations of subtilin were determined in a group of serial dilution tests carried out in the Dubos-Davis medium, which was modified for the purpose of this experiment only in its concentration of Tween 80. In Table I are presented the results of a representative experiment. As may be seen in Table I, in the control without Tween and in the lowest concentrations of Tween, the growth of tubercle bacilli was in the form of macroscopic clumps, and under these conditions the subtilin had little or no effect. The presence of Tween in concentrations sufficient to permit diffuse growth of the organisms resulted in a striking increase in the inhibitory action of subtilin, to the extent that growth was inhibited by 1.56 μg per ml. As previously mentioned, the description of the types of growth reported in Table I is based on macroscopic appearance. When viewed in a hanging drop preparation an even more striking difference was observable. In Fig. 1a may be seen clumped growth of tubercle bacilli in the absence of Tween, as seen in such a preparation. The clumps are seen to consist of many bacterial cells arranged in branching skeins or cords. This type of growth has been described in detail by Middlebrook, Dubos and Pierce.(9) For comparison, in Fig. 1b is shown a hanging drop preparation of diffuse or dispersed growth of tubercle bacilli in liquid synthetic medium containing 0.05% Tween. Its appearance was in striking contrast with clumped growth and may be seen to consist of a uniform single cell suspension of bacilli.

It has been previously noted that in the Dubos-Davis medium the action of Tween could be antagonized by the addition of serum and this phenomenon appeared to afford an additional means of examining the relationship between subtilin activity and the type of growth of tubercle bacilli. The following experiments were thus designed to test the effects of serum on this relationship. To Dubos-Davis medium modified to contain Tween in concentrations from 0.05% to 0.8%, serum was added to give final concentrations ranging from 2.5 to 20%. The inhibitory concentration of subtilin was then determined in each of these media and the type of growth of tubercle bacilli in each test noted. The results are shown graphically in Fig. 2. The percentage of serum and the percentage of Tween in the individual tests are expressed as the serum-Tween ratio. A low ratio reflects a low concentration of serum or a high concentration of Tween in a particular test. On the ordinate is plotted the inhibitory concentration of subtilin, and along the base is recorded the type of growth in the control tubes at the various ratios of serum and Tween.

As may be seen in Fig. 2, serum antagonized the action of Tween, so that when the serum-Tween ratio was high, growth was clumped and subtilin activity slight. As the ratio decreased, growth became diffuse and subtilin activity correspondingly increased. In these tests, it was observed that the effect of serum in concentrations as high as 20% was overcome by the presence of 0.4% Tween or more (serum-Tween ratio of 50 or greater), with resulting diffuse growth and a high degree of subtilin activity.

In Fig. 1 is shown the appearance of tubercle bacilli in representative cultures from this experiment as seen in hanging drop preparations. As may be seen, (Fig. 1c) in 20% serum growth was clumped if the medium contained 0.05% Tween, the usual concentrations employed, and appeared identical with the type of growth seen in liquid medium which contained no Tween (Fig. 1a). In the same concentration of serum, however, diffuse

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|| Preparations of subtilin were obtained through the courtesy of the Wallerstein Company, New York City, and from the Antibiotics Study Section of the National Institutes of Health, U. S. Public Health Service, Bethesda, Md. The latter preparation, Lot 200 A-1, was produced by the Western Regional Research Laboratory of the U. S. Department of Agriculture at Albany, Calif.

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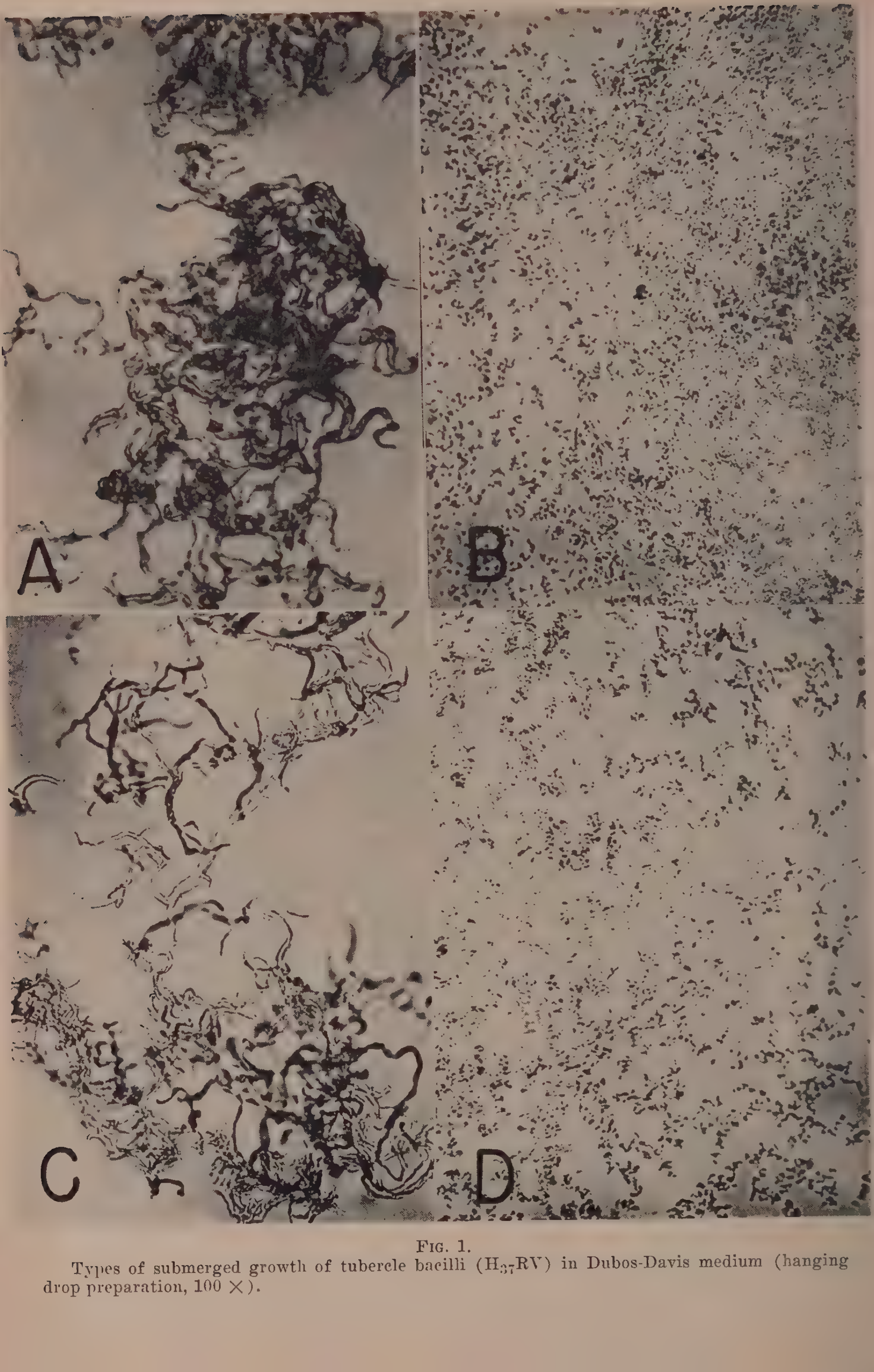

FIG. 1.
Types of submerged growth of tubercle bacilli ($H_{37}RV$) in Dubos-Davis medium (hanging drop preparation, 100 ×).

a. Clumped growth in medium containing *no Tween.* One may observe a ropy or corded appearance of the clumps.

b. Diffuse or dispersed growth in Dubos-Davis medium which contains 0.05% Tween.

c. Clumped growth in medium containing 0.05% Tween but, in addition, 20% serum. This type of growth strikingly resembles the growth in a, in which no Tween is present.

d. Diffuse growth in medium containing 20% serum. The Tween concentration, however, has been increased to 0.8%. This type of growth strikingly resembles that seen in b, in which Tween but no serum is present.

growth occurred (Fig. 1d) in 0.8% Tween, and resembled the type of growth customarily observed in the Tween-albumin medium with 0.05% Tween but without the antagonism of serum (Fig. 1b).

Further experiments were performed to discover whether this mechanism was sufficient to explain the failure of subtilin to be effective *in vivo.* Serum was obtained from rabbits at intervals following intravenous injection of 22 mg per kg of subtilin and was tested for inhibitory activity on *Str. hemolyticus* and *Staph. aureus.* Parallel assays of these sera were made with *M. tuberculosis* (H37Rv) as test organism, and were carried out in media containing 0.4% and 0.05% Tween as well as one without Tween. In Fig. 3, the results are recorded as the inhibitory concentration of subtilin in micrograms per ml. It may be seen that the activity of serum tested with tubercle bacilli in 0.4% Tween paralleled that found in assays with streptococci and staphylococci. There was no demonstrable inhibition of the tubercle bacilli in the two control media (not shown) containing 0.05% and no Tween.

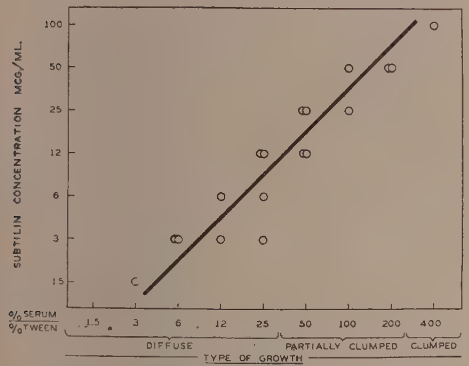

FIG. 2.

Effect of alterations in the ratio of serum and Tween on the inhibition of *M. tuberculosis* by subtilin.

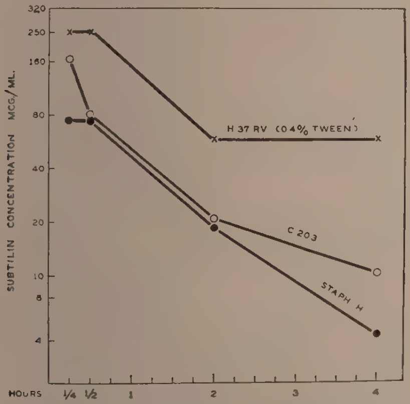

FIG. 3.

The antibacterial activity of serum of rabbits subsequent to intravenous administration of subtilin, as measured with 3 test organisms.

Discussion. It appears from these observations that the factors operative in the production of growth of tubercle bacilli in clumps or cords in a liquid medium afford these microorganisms a high degree of protection from the action of subtilin. Moreover, at least several other antimicrobial agents are considerably more active against tubercle bacilli which are growing diffusely than when the microorganisms are growing in clumps. This suggests the possibility that the clumped growth of tubercle bacilli reflects the presence of a mechanism which serves to protect the microorganisms from the harmful effects of certain antimicrobial drugs to which they would otherwise be sensitive. The question immediately arises as to the nature of the growth of tubercle bacilli *in vivo,* for if these findings may be more broadly applied, one might anticipate the failure of subtilin to affect tuberculosis in animals if the microorganisms existed as aggregate rather than as individual cells. Dubos has called our attention to the original reports of Koch(10) on the etiology of tuberculosis in which may be found draw-

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ings made from histological sections of necrotic foci of tuberculosis in man and animals. In these drawings the presence of tubercle bacilli in large aggregates is clearly illustrated. Moreover, Middlebrook, Dubos and Pierce(9) have also observed clumped growth of tubercle bacilli in the brain tissue of guinea pigs infected intracerebrally with virulent human strains. It is of interest that the occurrence of this phenomenon has not been more widely discussed. It is possible that modern methods of preparing tissues for histologic examination may not be ideally designed to reveal the nature of the type of growth of *M. tuberculosis in vivo.* On examination of conventionally prepared tissue sections of experimental tuberculous lesions of the lung, virtually all of the microorganisms during the early stages of the infection appear to be intracellular, principally within the monocytes. Under these conditions the tubercle bacilli are present as aggregates but the role of the phagocyte in producing the aggregation cannot be discounted. The principal situation where tubercle bacilli are seen to be extracellular is within areas of caseation where they are also present in clumps. In this situation, also, it is difficult to exclude the possibility that the aggregations were originally formed by the monocytes and were released with disruption of the latter cells.

Thus it cannot be considered as established at present that the clumping of bacilli observed *in vivo* has the same physiologic implications in regard to antimicrobial therapy as the clumping of bacilli *in vitro.*

Steenken and Wolinsky(11) have recently confirmed the observation that subtilin activity *in vitro* is dependent upon the presence of Tween (sorbitan monooleate). They further observed that guinea pigs infected with a virulent human strain, H37Rv, of *M. tuberculosis* were not benefitted by an extensive course of treatment with subtilin.

Summary. It has been observed that the degree of inhibition of tubercle bacilli by subtilin *in vitro* parallels the degree of growth dispersion of the organisms. In a medium permitting diffuse growth, subtilin was highly active. Conversely, in a medium in which growth was clumped, whether because of omission of Tween or from antagonism of Tween by serum, no significant inhibition of growth by subtilin was demonstrable. When serum from rabbits injected with subtilin was tested against tubercle bacilli in a medium containing sufficient Tween, the observed antituberculous activity paralleled the activity against streptococci and staphylococci. If the amount of Tween added to such tests was insufficient to produce diffuse growth of tubercle bacilli, subtilin activity was greatly diminished or absent.

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Relation of Low Temperatures to X-ray Injury of Hematopoietic Tissue of Tadpoles.* (17575)

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Tadpoles of *Rana catesbiana,* from 70-90 mm total length, were subjected to x-ray treatment of definite dosage and killed at the end of specific periods of time during which some were refrigerated and others kept at normal temperature. When tadpoles were refrigerated during irradiation, they were previously kept in the refrigerator for 2 hours. During irradiation, tadpoles were kept in water 7 mm deep, leaving the dorsal surface

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TABLE I.
% of Destruction in 24 Hours.

Exp. No.	Norm. temp., °C	Exp. temp., °C	No. of cases	Norm T. × 500 r norm. T.	Norm. T. × 500 r refrig.	Refrig. × 500 r refrig.	Refrig. × 500 r norm. T.
106	18.8	.0	33	(5) 21.3	(9) 0.6	(8) 0.9	(11) 10.8
99	18.8	.5	39	(16) 18.8	(5) 1.5	(11) 3.2	(7) 14.8
108	16.1	2.7	35	(5) 19.7	(10) 0.8	(11) 0.6	(9) 10.1
109	22.2	5.5	31	(5) 34.9	(9) 0.9	(8) 2.1	(9) 34.4

Figures in parentheses indicate numbers of tadpoles in each division within the experiment. We have extensive controls of unirradiated tadpoles kept at normal temperature and under these different degrees of refrigeration. Especial study was given to those kept at 0°C. Little or no injury resulted from refrigeration.

uncovered. The caudal portions of the mesonephroi were fixed in Bouin's fluid, cut at 5 μ and stained with Wright's or Leishman's blood stain. Counts of normal and dead hematopoietic cells were made with a Whipple grill under oil immersion, the numbers counted in each tadpole ranging from 275-450.

There were 4 types of treatment:

(1) Normal temperature before and during x-ray → Normal temperature.

(2) Normal temperature before and during x-ray → Refrigeration.

(3) Refrigeration before and during x-ray → Refrigeration.

(4) Refrigeration before and during x-ray → Normal temperature.

"Normal" temperature is that to which tadpoles were subjected in bowls kept in shade out-of-doors during August, September and October. The average temperatures for the dates of the experiments are given in the second column of the table. X-ray treatment was given with 250 KV, 15 m.a., .21 inherent Cu + .5 mm. Parab. Cu + 1 mm Al, h.v.l. 1.85 mm, Cu 35 cm distance F.O.D. Roentgens measured in air. Tadpoles placed in water leaving dorsal half exposed.

The results are shown in Table I.

A. X-ray irradiation of a dosage very destructive under normal temperatures can be prevented from producing appreciable visible effects on the hematopoietic cells if the tadpoles are maintained at 0° to 5.5°C.

B. Refrigeration during irradiation certainly does not prevent injury of hematopoietic tissue in tadpoles subsequently kept at normal temperatures. To what degree, if any, it may lessen the proportion of destruction is a subject for further study. In an experiment using 58 tadpoles, and extending from 3 to 4 days, we had similar results. Tadpoles irradiated and refrigerated for 2 days, then removed to normal temperature for 1 or for 2 days, showed very heavy cell destruction, while others treated identically and kept refrigerated at 2°C until killed at the end of 4 days, showed extremely little cell destruction. This will be fully discussed in our complete report.

Discussion. Our findings regarding the arrest of x-ray destruction of hematopoietic cells by refrigeration run parallel to the work of Patt,(1) Swift, Tyree and Clayton who showed that frogs that have been heavily x-rayed can be maintained alive for several months so long as they are kept refrigerated at 6°C. When transferred to normal temperature they quickly die.

These results might be due either to an arrest of metabolic activity or of mitosis. The work of Gluckmann and Spear(2) upon the arrest of mitotic activity in the germinal zone of the retina of tadpoles kept at low temperatures after gamma ray treatment would appear to favor the latter alternative. We have begun several lines of experimentation to solve this problem but the work is not far enough advanced to justify conclusions. The problem is quite complicated, indeed both factors might be involved. Tadpoles offer great advantages in experimental technics and in cytological studies of results.

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Summary. So long as tadpoles are maintained at temperatures from 0° to 5.5°C the destructive effects of x-ray exposure are very slight in the hematopoietic cells. The temperature of tadpoles at the time of x-ray treatment makes little if any difference in the destructive processes that develop later.

These destructive effects depend primarily upon the temperature to which the tadpoles are subjected after irradiation.

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Survival of Guinea Pig Thyroid and Parathyroid Autotransplants Previously Subjected to Extremely Low Temperatures.* (17576)

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Numerous investigations have been carried out on the effects of freezing and below freezing temperatures on infectious organisms and on the cells and tissues of plants, lower animals and cold blooded vertebrates, but relatively few studies have been reported in homotherms. In the latter group there have been reports of the survival of spermatozoa,(1-5) embryonal tissues,(6) the epithelium of chicken trachea(7) and the squamous epithelial cells of homologous skin grafts in the rat.(8) There are also numerous reports of the storage of tumor tissues at low temperature for periods up to two years with subsequent successful animal transfer.(7-21) Recently Gye and also Mann have stated that these results in tumors indicate the transfer of a tumor producing virus. Mann and Dunn(21) believe they have further support of this thesis in that they have been able to propagate mouse carcinoma by dried, frozen tumor tissue. However, the acceptance of such an interpretation rests, at least partly, on the proof that normal cells cannot survive similar treatment.

The present report presents data which indicate that normal mammalian tissues can survive exposure to extremely low tempera-

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† Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, St. Louis University.

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tures. Autotransplantation of previously frozen thyroid has been used to demonstrate survival, since the extensive investigations of Loeb(22) have shown that this type of transfer of normal (unfrozen) organs can be carried out successfully with consistent results. The thyroid gland was further chosen in order to utilize pituitary thyrotropin to increase the growth potential of the transplant and aid in overcoming the injurious effects of transplantation. Silberberg(23) has shown that thyroid autotransplants respond readily to such hormonal stimulus. Successful parathyroid transplants were incidentally encountered when inadvertently frozen and transferred with the thyroid tissue.

Method. 200-400 g guinea pigs were used in these experiments. Autotransplants were made into a subcutaneous pocket in the anterior abdominal wall. In some experiments 1 mg of thyrotropin‡ was administered subcutanously for 3-4 days prior to, or subsequent to transplantation. For storage at –70°C, the thyroid glands were inserted into 20 cc vials which were placed in contact with pieces of solid carbon dioxide in an insulated box. The thyroids were immersed directly into liquid nitrogen to obtain exposure to –190°C. In all of the freezing experiments no attempt was made to accelerate the decrease in temperature of the tissue.

Control Group. In a group of 5 guinea pigs immediate transplants from neck to subcutaneous tissue were carried out. One transplant was removed 24 hours later, another 5 days after transfer, 2 after 13 days, and the fifth after 20 days. Within the first 24 hours necrosis of much of the thyroid had taken place; there remained a few peripheral islands of viable thyroid follicles. By the end of the 5th day fibroblasts had started infiltrating the necrotic tissue, and by the 13th day had completely replaced it. After 20 days there was considerable regeneration of thyroid follicles so that islands of thyroid tissue partially surrounded the organized center. These follicles were lined by flat or cuboidal epithelium and contained pale scant colloid. A parathyroid was found in the 20 day transplant which showed a ring of well preserved epithelial cells surrounding a center of loose connective tissue in which there were a few phagocytes containing a brown granular pigment, presumably hemosiderin. Thyrotropin was administered to a second group of 3 guinea pigs. Two of these received the thyrotropin for 4 days before autotransplantation and the third for a similar period after transplantation. In the former group the transplants were examined after 5 and 11 days. There was less necrosis than in noninjected controls and the areas of preserved thyroid tissue were larger. The follicles were lined by columnar epithelium showing a number of mitoses. Fig. 1 is a section through the 11-day autotransplant. The guinea pig which received thyrotropin after transplantation also showed a well preserved transplant in which there were numerous mitoses even

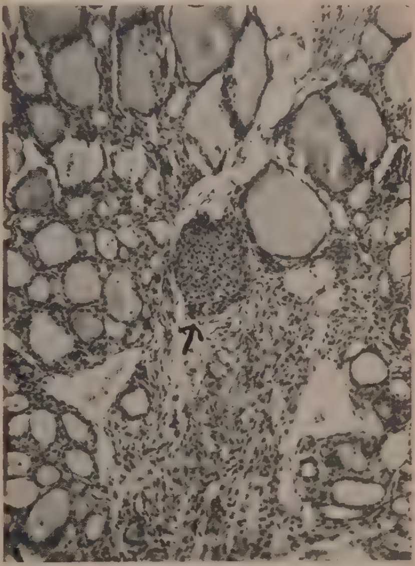

Fig. 1.

11-day autotransplant of thyroid and parathyroid. Guinea pig had received 1 mg of thyrotropin (Armour) daily for 4 days before transplantation. The thyroid follicles are well preserved and lined by cuboidal epithelium. Arrow indicates an area of parathyroid tissue.

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22. Loeb, Leo, *The Biological Basis of Individuality*, C. C. Thomas, Springfield, Ill., 1942.

23. Silberberg, M., *Arch. Path.*, 1934, v17, 381.

‡ We are indebted to The Armour Laboratories, Chicago, Ill., for supplying the thyrotropin used in these experiments.

as late as 11 days after the last injection.

In order to observe the immediate effects of freezing, one guinea pig received thyrotropin for 3 days, following which its thyroids were removed; one was placed at –70°C for 24 hours, and the second at –190°C for 10 minutes. The glands were then thawed, fixed and sectioned. In both the architecture was well preserved, although the colloid was soft, pale and sparse; there were many pyknotic nuclei. However, the thyroid kept at the lower temperature showed better preserved nuclei and several mitotic figures were found.

Experimental Group. Nine lobes of thyroid were autotransplanted after being kept at –70°C for 24 hours; they were examined after periods varying from 5-20 days. All except 2 of these transplants showed only progressive organization of necrotic tissue (Fig. 2). However, in one transplant examined after 13 days there was a well preserved parathyroid gland with a central area of loose connective tissue containing a few phagocytic cells with brown granular pigment (Fig. 3); this was similar to a parathyroid observed in a control autotransplant. Another 13 day transplant contained an area of well preserved thyroid tissue adjacent to organizing necrotic tissue (Fig. 4). The epithelium of the follicles was flat and the colloid soft, pale and sparse; a mitosis was found in one section. In a further experiment one guinea pig received thyrotropin for 4 days prior to removal of a lobe of thyroid which was then subcooled to –70°C for 24 hours and autotransplanted. In 2 other guinea pigs the pituitary hormone was administered for 4 days after subcooling to this temperature and autotransplantation. The first transplant was examined after 11 days, and the latter 2 after 15 days; no preserved thyroid tissue was found in any of these transplants.

Seven guinea pigs received daily injections of thyrotropin for 3 or 4 days, following which a lobe of thyroid was removed, placed at –190°C for 10 minutes and then autotransplanted. Four of these transplants were removed after 3 days when the animals were found dead. The transplants, however, were extremely well preserved; there was no necro-

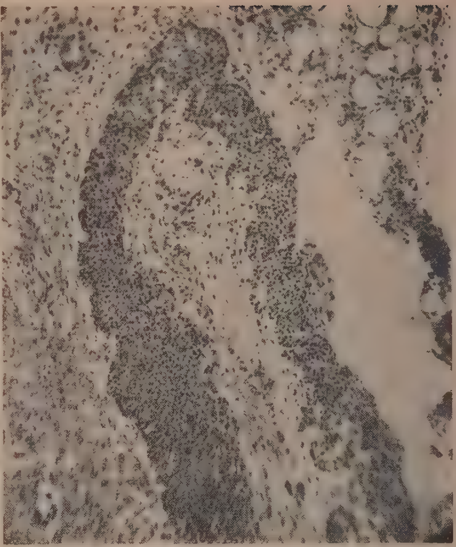

FIG. 3.
13-day parathyroid autotransplant following exposure to –70°C for 24 hr. The parathyroid epithelium forms a ring about a central core of connective tissue.

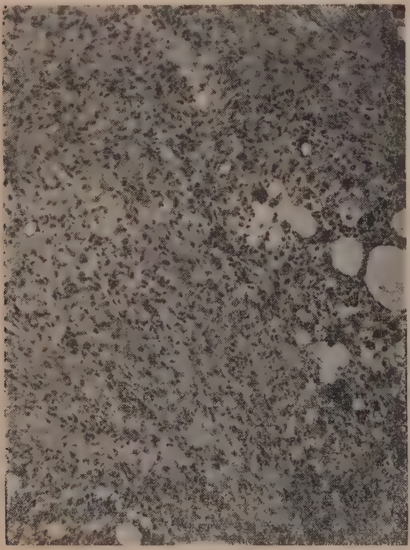

FIG. 2.
14-day unsuccessful thyroid autotransplant following exposure to –70°C for 24 hr. The thyroid tissue has been completely replaced by fibroblasts. The giant cells represent a reaction to necrotic transplanted fat.

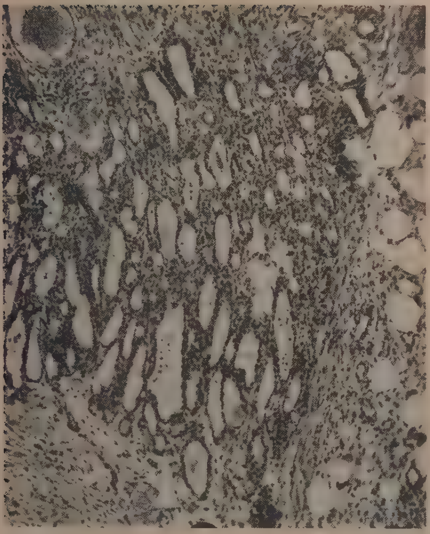

FIG. 4.
13-day successful autotransplant of thyroid following exposure to −70°C for 24 hr. The thyroid follicles are small and contain very little colloid. The epithelial lining is cuboidal in character.

sis, although some of the nuclei were pyknotic; the epithelium lining the follicles varied from a cuboidal to columnar character and several mitoses were found (Fig. 5). Two other guinea pigs were found dead on the fifth day after autotransplantation; although there was some central necrosis in these transplants, there were well preserved peripheral groups of follicles lined by cuboidal epithelium. The fifth transplant was removed after 11 days and showed complete fibroblastic organization.

Finally an experiment was performed in which two temperatures were employed. The thyroids were first immersed in liquid nitrogen for 10 minutes and then transferred to −70°C where they remained for 24 hours when they were autotransplanted. There were 4 guinea pigs in this group. One guinea pig received thyrotropin for 4 days after autotransplantation, and on the fifth day the transplant showed a well preserved thyroid with follicles lined by cuboidal epithelium; the colloid was sparse and pale staining. The remaining three guinea pigs received thyrotropin before the glands were frozen and autotransplanted. Two of these transplants were examined after 5 days and showed necrosis and fibroblastic organization of the periphery of the transplant, but the third thyroid which was examined after 11 days showed excellent preservation (Fig. 6). The follicles were lined by cuboidal and columnar epithelium; occasional mitoses were found. The colloid was pale and sparse. There was some fibroblastic reaction in the surrounding fat, accompanied by an occasional multinucleated cell, but this reaction was common to many transplants, both successful and unsuccessful, and represents a reaction to transplanted fat frequently injured in the process of inserting the transplant into the subcutaneous pocket.

Discussion. As far as we have been able to determine, these experiments represent the first successful demonstration of the survival of a functional structural unit of an adult homotherm following exposure to extremely low temperatures. Two reports previously cited give evidence of survival of cells other

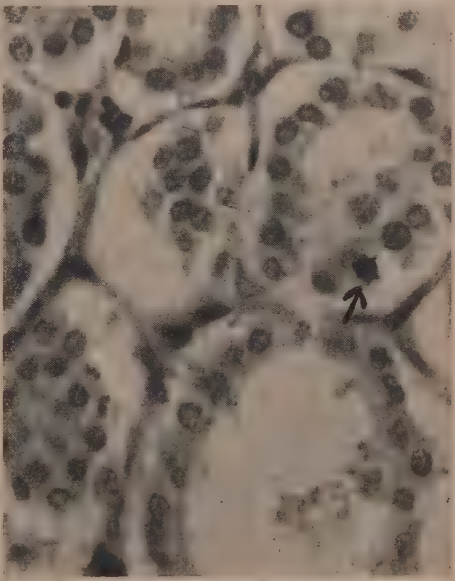

FIG. 5.
3-day autotransplant of thyroid. Treatment previous to transplantation consisted of the injection of 1 mg of thyrotropin daily for 3 days followed by immersion in liquid nitrogen (−190°C) for 10 minutes. The epithelium of the follicles shows an excellent state of preservation and retains a hyperplastic character indicative of a response to thyrotropin. Arrow indicates a mitotic figure.

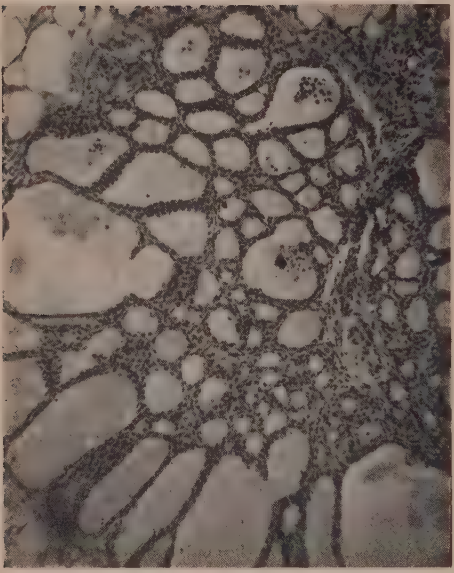

FIG. 6.
11-day autotransplant of thyroid. Treatment previous to transplantation consisted of the injection of 1 mg of thyrotropin daily for 3 days followed by immersion in liquid nitrogen (–190°C) for 10 minutes and storage at –70°C for 24 hr. Note excellent preservation of follicular structure.

than spermatozoa of adult warm blooded animals so treated. In one of these the criterion of viability was movement of the cilia of respiratory epithelium (Breedis and Furth) (7), while in the second there was evidence of mitotic activity in the homologously transplanted epithelial cells of the rat's skin.(8) In the first instance the observation may be criticized on the basis that ciliary movement may have been caused artificially by air or liquid currents; one of these investigators himself subsequently raises objections to movement as a criterion of survival of cells.(18) The criterion of growth as represented by mitotic activity is a much more accurate indicator of viability; such evidence is present in the investigations of Mider and Morton(8) and in our experiments.

There are contradictory reports in the literature as to whether rapid or slow freezing is less injurious to cells. The primary factor in producing death in frozen tissue is apparently the formation of ice crystals.(24,25) Luyet and Gehenio(24) have divided the organisms which resist cold into two groups, namely (1) those that resist cold only in a dry state, and (2) those that resist cold with their full water content. Of the latter, some organisms survive only if special precautions for rapid cooling and rewarming are taken in order to prevent the formation of ice crystals. The critical range of temperature in subcooling tissues would therefore be that span in which ice crystals may form. If this range is passed through rapidly, a vitreous state (Luyet and Gehenio) results which is apparently not injurious to cells in most instances, and in which state they probably may be preserved indefinitely. On the other hand, several investigators report better results in tumors(7,8,18) and in homologous transplants of rat's skin(8) with slow freezing. Breedis (18) explains this on the basis that the initial freezing of water occurs in the extracellular fluids which then results in the concentration of osmotically active material outside of the cell. It is believed that this results in partial dehydration of the protoplasm which would then become a less favorable site for ice crystal formation. Since in the present experiments considerably better results were obtained with rapid immersion in liquid nitrogen it would appear that these experiments tend to substantiate the observations of Luyet and Gehenio. However, it is possible that different tissues respond differently after exposure to low temperatures.

We have pointed out in the introduction that the successful transfer of frozen tumor material is believed by some investigators to indicate the transfer of a virus, since they assume that normal and malignant cells cannot survive exposure to freezing and below freezing temperatures. The present studies show that this is not the case. Nor does the successful transfer of frozen, dried tumor material necessarily prove the presence of a virus. The investigations of Luyet and Gehenio(24) indicate that dehydration may facilitate rather than impede survival of tissues at extremely low temperatures.

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Summary. Thyroid gland of guinea pig was subjected to low temperatures (–70°, or –190°C) and subsequently autotransplanted. In some instances pituitary thyrotropin was administered before or after transplantation in order to increase the growth potential of the transplant and overcome the injurious effects incident to transferring the tissue. There was one successful transplantation of thyroid gland in 12 attempts after exposure at –70°C and one parathyroid transplant. Eight viable thyroid grafts in 12 attempts were obtained after immersion at –190°C. The significance of these findings with respect to rapidity of freezing is discussed in their relation to the mechanism of survival of mammalian cells and tissues exposed to extremely low temperature. The relation of these results to evidence purported to indicate a viral transmission of transplanted frozen-dehydrated tumor material is also pointed out.

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Comparative Study of Agglutinins and Complement-Fixing Antibodies for Brucella in Blood and Cerebrospinal Fluid. (17577)

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Although brucellosis commonly causes symptoms referable to the nervous system, objective evidence of the results of invasion of the central nervous system is frequently lacking. Over a period of 12 years at the University of Minnesota and cooperating hospitals, a total of 161 cases of active brucellosis, 86 of which were culturally proved, have been studied, and the diagnosis of encephalomeningitis was made in four cases.(1) A fifth case exhibited a severe cerebral vascular accident secondary to subacute bacterial endocarditis, which was due to *Br. abortus*.(2) Except for the cases cited, there was no evidence of organic cerebral changes in the remaining cases, although psychic disturbances with emotional instability and mental depression were commonly encountered. Apter and his associates(3) were impressed by the "disturbances in the adaptive capacities" of patients having chronic brucellosis and carried out a battery of neuropsychologic studies, as outlined bp Halstead,(4) on a series of 10 patients. As a result of their investigations, it was concluded that organic damage to the frontal lobes had occurred in 7 of the 10 patients. While such a study suggests a reasonably high incidence of brain damage in patients with chronic brucellosis, there remains an urgent need for acquiring more information on the frequency with which the central nervous system is invaded by *Brucella*. Since serologic techniques are applied successfully to an examination of the cerebrospinal fluid in individuals with suspected syphilis, there was a possibility that serologic methods utilized for the diagnosis of brucellosis might be of some aid in resolving the problem of neurobrucellosis. Simultaneous tests on the blood and cerebrospinal fluid of patients with brucellosis were performed, utilizing procedures for detecting the presence of agglutinins and complement-fixing antibodies.

Methods. Specimens of venous blood and

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* This investigation was supported in part by a research grant from the National Institutes of Health.

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TABLE I.
Comparative Results of Agglutination and Complement Fixation Tests on Blood and Cerebrospinal Fluid.

Case No.	Duration of illness	Titer of agglutinins		Titer of complement-fixing antibodies	
		Blood	CSF	Blood	CSF
A. Patients with *Br. abortus* recovered from blood or cerebrospinal fluid					
1	6 mo.	160	Neg.	20	Neg.
		1280	”	10	”
2	2 yr	640	10		
3	3 ”	640	2	320	4
4	4 ”	640	320	160	128
B. Patients with illness due to brucellosis but with sterile cultures					
5	2 mo.	640	Neg.	40	Neg.
6	3 ”	640	”		
7	5 ”	320	”	2.5	”
8	6 ”	2560	”	320	”
9	1 yr	640	”	160	”
10	1 ”	640	”	40	”
11	2 ”	320	”	20	”
12	3 ”	160	”	5	”
13	6 ”	320	”	5	”

cerebrospinal fluid were obtained from the patients on the same day. Tests for the presence of agglutinins and complement-fixing antibodies were determined simultaneously on the blood and cerebrospinal fluid. In performing the agglutination test, the macroscopic tube method was employed in which falling concentrations of serum up to a dilution of 1 to 5120 were used. The antigen was obtained from the Bureau of Animal Industry, Beltsville, Md., and was prepared from Strain 1119-3 of *Br. abortus*. This antigen has proved satisfactory for diagnostic purposes over a period of several years in the Laboratory of the Minnesota State Department of Health and at the University Hospitals. Mixtures of the serum or cerebrospinal fluid and antigen were incubated overnight at 37°C. for 18 hours, and then placed in a refrigerator at 4°C. for one hour. The tubes were then examined macroscopically. Antigen for the complement-fixation test was obtained from Dr. C. W. Bonynge of Los Angeles, Calif. This Brucella antigen had proved to be quite satisfactory for detecting complement-fixing antibodies in bovine and human serum. The Kolmer-Wassermann(5) technique for detecting complement-fixing antibodies in syphilis was used, substituting the Brucella antigen.

Results. Studies were carried out in 13 patients with brucellosis, and the results are presented in Table I. In group A, there were 4 patients from whom *Br. abortus* was obtained. Cases 2 and 4 were instances of encephalomeningitis with isolation repeatedly of *Br. abortus* from the cerebrospinal fluid. The fluid in both cases showed an exceedingly high protein content with an increase in cells and a reduction in sugar. Although in case 2 Brucella agglutinins were found in the spinal fluid in a titer of 10, unfortunately no test for complement-fixing antibodies was carried out. Case 4 had had evidence of chronic encephalomeningitis for 4 years. Evidence of diffuse cortical damage was demonstrated in the electroencephalogram, and he had acquired complete nerve deafness. It was in this patient's fluid that a high titer of Brucella agglutinins and complement-fixing antibodies was demonstrated. Case 3 was of interest in that the patient had been ill for 3 years. *Br. abortus* was isolated from blood cultures, but the cerebrospinal fluid remained sterile. A complete examination of the fluid removed from the lumbar area revealed 2 mononuclear cells

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5. Kolmer, J. A., and Boerner, F., *Approved Laboratory Technic*, Fourth Edition, New York, Appleton Century Co., 1945.

and normal protein and sugar values. A paravertebral abscess was demonstrated roentgenologically in association with a destructive process of the seventh and eight thoracic vertebrae and the intervening intervertebral disc.

There were 9 patients in Group B. Although brucellosis was not proved bacteriologically in these cases, the clinical course and the titer of Brucella agglutinins in each of the cases was compatible with the diagnosis of brucellosis. Many of these patients had somatic complaints. The nature and chronicity of their symptoms were consistent with those observed in psychoneurotic individuals. It was in this group of cases that serologic data were highly desired. But in every instance antibodies were absent in the cerebrospinal fluid by both technics. Case 13 was of particular interest in that he had contracted his illness 6 years before being studied. He had had narcolepsy for 3 years. An electroencephalogram was within normal limits, as were the findings in his cerebrospinal fluid.

Discussion. Most authorities agree that for diagnostic purposes with human serum in brucellosis the complement fixation test does not possess any advantage over the agglutination test.(6) The present study indicates that this also applies to cerebrospinal fluid. In their review on neurobrucellosis, Roger and Poursines(7) point out instances of meningitis with Brucella agglutinins present in the cerebrospinal fluid in a low titer. In our own experience, the presence of Brucella antibodies in the cerebrospinal fluid has been associated with demonstrable organic changes such as encephalomeningitis or spondylitis. Perhaps further attention should be given to cultural studies of cerebrospinal fluid, especially in patients with chronic brucellosis having psychosomatic disturbances. In the cases of encephalomeningitis (cases 2 and 4), *Br. abortus* was cultured from the cerebrospinal fluid with the aid of tryptose phosphate broth. Goodpasture and Anderson (8) first called attention to the marked susceptibility of viable embryonic chicken tissue to *Brucella.* This was subsequently confirmed by Hall and Spink.(9) In a limited number of cases of human brucellosis, Dr. Wendell Hall attempted to isolate *Brucella* from the cerebrospinal fluid of patients having a demonstrable bacteremia. He succeeded in growing *Br. abortus* from the tissues of 11 day old chick embryos after apparently normal cerebrospinal fluid from a patient with chronic brucellosis was injected into the allantoic sac. He repeated the results with another specimen of fluid from the same patient. Subsequently it was observed that this patient had Brucella spondylitis. Specimens of cerebrospinal fluid from this patient introduced simultaneously into tryptose phosphate broth showed sterile cultures.

Summary. Simultaneous tests for agglutinins and complement-fixing antibodies for *Brucella* were carried out on the serum and cerebrospinal fluid of 13 patients having brucellosis. Complement-fixing antibodies and agglutinins were demonstrated in the cerebrospinal fluid of one patient with chronic encephalomeningitis and in one patient with a paravertebral abscess associated with Brucella spondylitis. A third patient having chronic Brucella encephalomeningitis had agglutinins in the cerebrospinal fluid. In 13 cases these two serologic tests when performed with cerebrospinal fluid have not aided in resolving the problem of the incidence of organic brain damage in patients with brucellosis.

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Estrogen-Inactivation by the Liver as Modified by Dietary Protein.* (17578)

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One of the functions of the liver is to inactivate both endogenous and exogenous estrogens. This phenomenon has been amply confirmed in both *in vitro* and in the intact animal.(1-5) The inactivation mechanism becomes impaired in the presence of hepatic damage in the experimental animal and in man.(6-9) Golden and Sevringhaus(10) implanted ovaries into a site drained by the hepatic portal system and demonstrated that if the liver is the first organ in contact with the estrogen, the latter is completely inactivated. Biskind and Marks(11) confirmed this observation by implanting pellets of estrone into the spleen. Biskind and Biskind (12) carried out further studies to show that when rats are placed on diets deficient in the components of the vitamin B complex the liver loses its ability to inactivate the estrone absorbed from the implanted pellet. Segaloff and Segaloff (1944)(13) claimed that the important components of the B complex in this respect were thiamine and riboflavin. Drill and Pfeiffer(14) and Jailer(15) although employing different methods, have demonstrated that the concomitant inanition which accompanies vitamin B deficiency is the important factor in the loss of ability of the rat liver to inactivate the estrogens in these experiments.

The purpose of this study was to ascertain whether the protein content of the diet can modify the role of inanition in the inactivation mechanism of estrogens by the liver.

Methods and Materials. Adult female rats of the Wistar strain were ovariectomized and pellets of α-estradiol weighing 1-3 mg were subsequently inserted under the capsule of the spleen. Only those animals whose vaginal smears reverted to the diestrus type within eight days were used as experimental animals. About one-fourth of the animals were discarded because the vaginal smears remained cornified. Vaginal smears were done daily throughout the course of the investigation and at the conclusion of the experiment the animals were sacrificed, the uteri dissected clean of connective tissue and weighed on a torsion balance.

Two different categories of experiments were performed. In the first, the animals were placed on iso-caloric diets deficient in the components of the vitamin B complex containing either 5, 15, or 50% casein. These rats were allowed to eat *ad libitum* and the

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† Present address: Department of Medicine and Obstetrics and Gynecology, College of Physicians and Surgeons, Columbia University.

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TABLE I.
Composition of Diets Used.

	5% protein	15% protein	50% protein
Casein	5	15	50
Sugar	81	72	38
Vegetable oil	8	8	8
USP salt mixture	4	4	4

amount of food consumed daily was recorded. The constituents of the diet are presented in Table I. Under this regimen the daily food intake gradually declined so that after 3 weeks the amount of food consumed was less than one-third that of the control period. In the second experiment, the same diets were employed, however the various components of the B complex (Vifort-0.3 ml every other day) were added to the drinking water. In this experiment the food intake was limited to 3 g daily.

Results. Vitamin deficiency experiments. In general, the vaginal smears in the animals on the 5 and 15% protein diets began to show signs of cornification on about the twentieth day while those on the 50% protein diet remained atrophic. On the twenty-seventh day after the institution of the diet the animals were sacrificed. The results are presented in Table II. The average uterine weights of those animals on the 50% casein diet showed practically no evidence of stimulation, while the uteri of the rats in the other 2 groups showed definite signs of estrogen activity. All 3 groups of animals consumed about the same quantity of food and lost about the same percentage of the initial weight on the vitamin B deficient regimen (Table II).

Inanition experiments. Although the intake of the components of the B complex was adequate, the results of this experiment were similar to those of the previous one (Table III). Here again, those animals which were on the 50% casein diet exhibited lower uterine weights than those on the 5 and 15% diets. This is indicative of a more efficient estrogen inactivation mechanism. It is also clear that in the 5 and 15% casein group there is some inactivation of the estrogen absorbed from the splenic pellet since the control group with intramuscular pellets showed still higher uterine weights (Table II). However, sufficient estradiol escapes inactivation by the liver so that there is approximately a 100% increase in uterine weight over those of the castrate controls.

Discussion. The data presented confirm the previous observations of Drill and Pfeiffer (14) and Jailer(15) that the inanition which accompanies vitamin B deficiency is an important factor in the inability of the liver to inactivate estrogens. There was no difference in the group of animals exhibiting avitaminosis B and the group on an inanition diet but receiving adequate amounts of the various components of the B complex. The animals in both groups of experiments were fed iso-caloric diets, consumed approximately the same amount of food and lost about the same amount of weight. Yet, the livers of those animals which received the 50% protein could inactivate practically all the estradiol absorbed from the pellet which had been implanted into the spleen. Only those rats which were maintained on the 5 and 15% protein diets, however, showed increased uterine weights, indicating that their livers have lost the ability to inactivate all the estradiol absorbed. Although the livers were not analyzed for their protein content, it is probable that the protein concentration varies directly with the protein content of the diet. The enzymes responsible for the degradation of estradiol or estrone have not been isolated as yet, but it would appear that they are proteins or at least the prosthetic groups may be attached to a protein. It is entirely possible that during inanition this enzyme cannot be synthesized by the liver, except in those animals which were consuming the 50% protein diet.

In both experiments the caloric intake of the animals was approximately one-third of that consumed by control animals allowed to eat *ad libitum.* Therefore, the animals consuming the 50% casein diet are getting the same absolute amount of protein as do the control animals (15% casein) although the total caloric intake is reduced. This appears to be a sufficient amount of protein to synthesize the enzymes responsible for the inactivation of estradiol. Unna *et al.* (1943) (16) have previously shown that liver slices of rats maintained on a low protein diet were

TABLE II.
Vitamin B Deficient Diet. Food Intake Unrestricted.

% protein diet	No. of rats	Wt, g		Food intake, g		Uterine wt, mg
		Start	Finish	Total	Protein	
5	6	166	104	159	8.0	99 ± 19
15	5	173	118	149	22.4	130 ± 27
50	6	148	109	159	79.4	66 ± 3
Castrate control	3					63 ± 3
Intramusc. pellet	4					157 ± 23

TABLE III.
Food Restricted to 3 Grams Daily. Vitamin B Complex Added.

% protein diet	No. of rats	Body wt, g		Uterine wt, mg
		Start	Finish	
5	7	186	112	118 ± 9
15	7	189	116	93 ± 9
50	8	186	117	70 ± 5
50 (No pellet)	5	209	125	50 ± 1

not as effective in inactivating estradiol *in vitro* as slices from the control rats. Hepatic damage whether surgically, chemically or nutritionally induced, interferes with the hepatic mechanism concerned in the degradation of the estrogens. György(9) has also shown that when rats are placed on a low protein diet the liver loses its ability to inactivate estrogens, the type of injury involved in his experiments is different from the ones reported here. His animals were fed a diet low in protein but high in fat which ultimately results in fatty infiltration and finally hepatic cirrhosis. Visible hepatic damage is present at the time the estrogen inactivation mechanism is impaired. In these experiments, on the other hand, there is no visible evidence of hepatic damage.

Summary. The liver of rats maintained on a 50% casein diet retains its ability to inactivate estradiol in spite of avitaminosis B or inanition. However, animals on a 5 or 15% diet under the same conditions cannot inactivate estradiol.

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Reduced Absorption of Aureomycin Caused by Aluminum Hydroxide Gel (Amphojel). (17579)

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Aureomycin given orally is effective in many microbial infections. The simultaneous oral administration of aluminum hydroxide gel and aureomycin will in some instances reduce the epigastric distress, nausea, and vomiting that occasionally occur when aureomycin is given alone. It has been recommended that this antibiotic should be prescribed along with a preparation of aluminum hydroxide suspension.(1,2) However, it has been found that solutions of aureomycin had very little antibacterial activity following exposure to aluminum hydroxide gel.(3) This observation suggested that if aluminum hydroxide were administered concurrently with aureomycin, similar loss of activity might occur in the bowel and greatly diminish the anticipated levels of the drug in the blood and tissues. The present report is therefore concerned with the influence of an orally administered aluminum hydroxide suspension† upon the absorption of aureomycin as measured by aureomycin blood levels obtained before and after aluminum hydroxide was instituted.

Method of Study. Daily levels of aureomycin in the serum of 5 hospitalized patients and 6 normal male subjects were determined. Aureomycin was administered for 3 consecutive days. This was followed immediately by a 3-day period during which aureomycin and aluminum hydroxide suspension were both administered. The dose of aureomycin was 500 mg taken orally every 6 hours. Two tablespoons of aluminum hydroxide were given immediately after each dose of aureomycin. Blood for the serum levels was drawn with sterile technique each morning and promptly refrigerated. The serum was separated by centrifugation within 3 hours after the blood was obtained and placed in a deep freeze unit where it was stored at –4°C. Aureomycin levels were determined by a modification of the broth dilution method of Dornbush.(4) Bacillus No. 5,‡ a strain of *Bacillus cereus*, was used as the test organism. Two-fold dilutions of the sera were made in broth, and .5 ml of a 1:100 dilution of an 18-hour broth growth of the organism was inoculated into each tube. The mixture was incubated for 4 hours at 37°C. The concentrations of aureomycin in the sera were calculated by multiplying the last dilution of serum that inhibited visible growth of the test organism by the sensitivity of that organism as determined at the same time. The sensitivity of the test organism varied from .01 mg per ml to .039 mg per ml. All the sera from each individual were examined simultaneously in order to avoid the effect of slight daily variation in the sensitivity of the test organism.

Results. In 4 patients, there was a fall in the level of aureomycin in the serum within 24 hours after they started to take aluminum hydroxide gel with aureomycin. After 48 hours of the combined medication, all serum levels were less than one mg per ml. Aureomycin without amphojel produced levels which averaged 5.9 mg per ml and ranged from 1.2 to 10.2 mg per ml. The fifth patient maintained a level of 5 mg per ml in spite of the aluminum hydroxide. One patient suffered a recurrence of her urinary tract infection on the third day of combined treatment which promptly subsided when the aluminum hydroxide was discontinued.

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* Aided by a grant from Lederle Laboratories.

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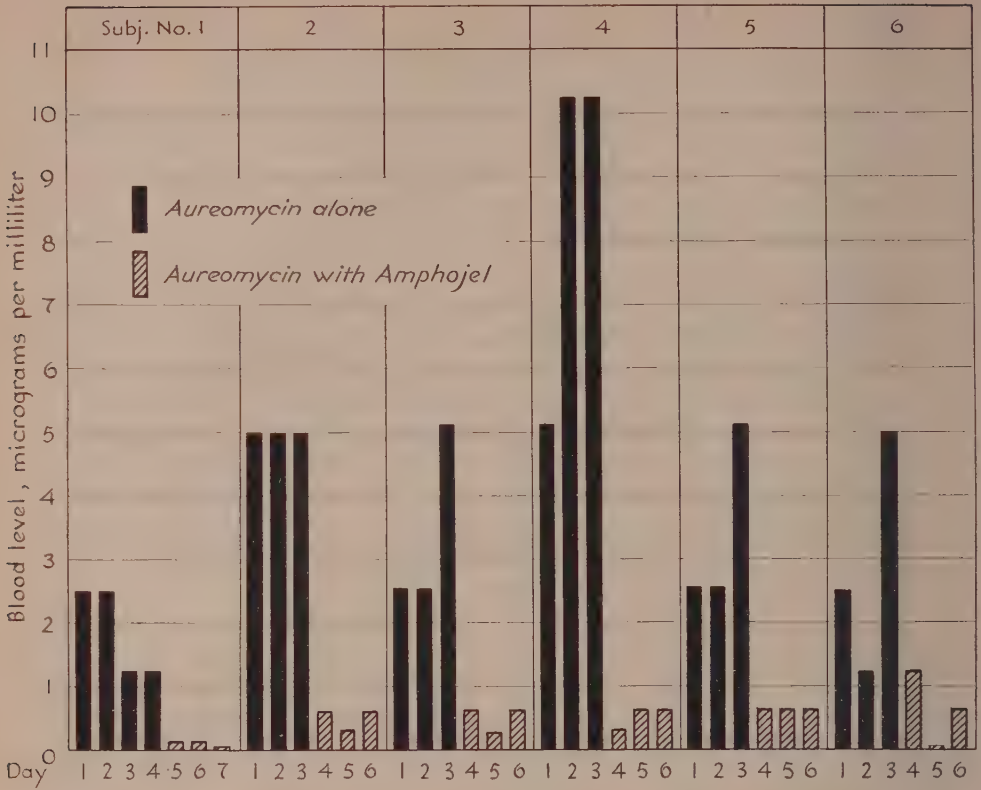

FIG. 1.

Serum levels of aureomycin in 6 normal subjects who first took aureomycin alone and then aureomycin and amphojel together.

The addition of aluminum hydroxide gel was followed by a sharp drop in the serum concentration of aureomycin in each of the 6 normal males. This drop occurred within 24 hours after they began to take aluminum hydroxide gel (Fig. 1). Levels before aluminum hydroxide gel averaged 4.2 mg per ml and ranged from 1.25 mg per ml to 10.24 mg per ml. After aluminum hydroxide gel, the average level was .49 mg per ml with a range of from .036 mg per ml to 1.25 mg per ml.

Four of the normal male subjects complained of loose unformed stools during the 6 days they were taking aureomycin. Three of them noted pruritus ani that disappeared after aureomycin was discontinued.

Discussion. These results clearly indicate that when aluminum hydroxide gel is given with aureomycin, a lowered level of aureomycin is obtained. This lowering of the serum level of aureomycin by aluminum hydroxide gel may explain some therapeutic failures in infections that usually respond to aureomycin. A possible explanation of these findings is that the colloidal particles of the aluminum hydroxide gel may adsorb the aureomycin and prevent its absorption through the gut. The possible influence by other foods and drugs on the absorption of aureomycin is worthy of further investigation.

Summary and Conclusions. Aluminum hydroxide gel (amphojel) reduced the level of aureomycin in the serum of 10 individuals within 24 hours after the gel was added to each dose of aureomycin. These findings indicate that the two drugs should not be used together.

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Effect of Different Levels of Hexahomoserine on Growth and Hematopoiesis in Rats. (17580)

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In a previous report(1) it was shown that young pigs stop growing and develop anemia when small amounts of hexahomoserine (α-amino-ϵ-hydroxy caproic acid) are added to an otherwise normal mixed-grain ration. The complete inhibition of growth of the pigs after ingestion of the compound for only one week was unexpected in view of the low level fed in the diet (3 g DL-hexahomoserine daily). To obtain more information on the properties of this compound, groups of white rats have been maintained for 28 days on diets containing different levels of hexahomoserine. In these experiments a separation of the growth-retarding and anemia-producing effects was observed.

Experimental. DL-hexahomoserine was synthesized from dihydropyran‡ by the method of Gaudry.(2) DL-hexahomoserine and DL-lysine monohydrochloride replaced equivalent weights of dextrin in the lysine-deficient basal zein ration shown in Table I. Young white rats from the stock colony were divided into groups of 6. Each litter was distributed as evenly as possible among the experimental groups, one rat to each group, and each group contained 3 males and 3 females. The rats were housed in individual cages with wire screen floors, and were fed *ad libitum*. The animals were weighed every fourth day. On the 28th day red blood cell counts(3) were made on blood from the tip of the tail, and the animals were then autopsied.

Results. The effect of the different levels of hexahomoserine on weight gain and red blood cell count is summarized in Table II. The average growth curves for animals in Groups I-VII inclusive, made by plotting the average total weight gains of each group at 4 day intervals, all have a positive slope characteristic for the level of lysine, or lysine and hexahomoserine, fed. In Group VIII, the curve reached a maximum (3 g) at the end of 8 days and then flattened out. The growth curves are not reproduced here.

The data in Table II show that the lowest level of DL-hexahomoserine fed (0.083%-Group IV) produced an average total weight gain (53 g) which is 80% of that of the control animals (Group I) receiving the same level of lysine without hexahomoserine. Increasing increments of hexahomoserine produced further reductions in average total gain. Statistical analysis§ of the average total gains in Groups IV-VII inclusive was made by plotting a regression curve using the percents of DL-hexahomoserine as the abscissae and the average total gains as ordinates. The regression of gain on amount of hexahomoserine was negative and highly significant, and appeared to be linear from 0 to 0.33% DL-hexahomoserine. Extrapolation of the curve to zero gain intersected the X axis at 0.6% DL-hexahomoserine. This level is about half way between the levels of DL-hexahomoserine fed to the animals in Groups VII and VIII.

To determine the effect of low levels of lysine alone on the growth rate and red blood cell count, the rats in Groups II and III were fed the basal zein diet supplemented with 1.5% and 1.0% of DL-lysine · HCl, respectively. At the 1.5% level (see Table II) the

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* Present address: General Foods, Inc., Hoboken, N.J.

† Journal paper No. 435, Purdue University Agric. Exp. Sta.

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§ We are indebted to Professor S. R. Miles for the statistical analyses.

TABLE I.
Composition of Basal Ration.

Component	%	Vitamin mixture Component	Mg per 100 g basal ration
Zein	26.1	Thiamin hydrochloride	.3
Dextrose	27.6	Riboflavin	.3
Dextrin	26.49	Calcium pantothenate	.5
Lard	11.0	Nicotinic acid	3.0
Mineral mixture(5)	5.2	Pyridoxine hydrochloride	.5
Cellufiour	2.0	Choline chloride	100
Liver extr. (Lilly)	.5	Inositol	20
DL-tryptophan	.4	Para-amino benzoic acid	5
Glycine	.13	Biotin	.01
L-histidine · HCl	.20	Folic acid	.01
Vit. mixture	.38	Vit. K (Menadione)	.50
		Nopco XX*	250
	100		
			380.12

* Contains 3,000 I.U. of Vit. A and 400 I.U. of Vit. D_2 per g.

TABLE II.
Effect of DL-Hexahomoserine on Growth and Red Blood Cell Count.

Group	Diet,* %	Avg total gain (28 days) g	Avg RBC count (28th day) cells × 10^6/cmm
I	2 DL-lysine · HCl	66	6.1
II	1.5 ” ”	52	6.1
III	1.0 ” ”	21	5.4
IV	2.0 ” ” + 0.08 DL-hexahomoserine	53	5.9
V	2.0 DL-lysine · HCl + 0.11 DL-hexahomoserine	51	6.0
VI	2.0 DL-lysine · HCl + 0.17 DL-hexahomoserine	49	5.7
VII	2.0 DL-lysine · HCl + 0.33 DL-hexahomoserine	32	5.7
VIII	2.0 DL-lysine · HCl + 1.0 DL-hexahomoserine	3†	3.9†

* The lysine and hexahomoserine each replaced an equivalent weight of dextrin in the basal zein ration (see Experimental Section).

† One rat died on the 24th day and is not included in the average.

rats made an average total gain that is approximately the same as that made by rats receiving 2% DL-lysine · HCl and 0.08-0.17% DL-hexahomoserine. At the 1.0% level (Group III) the rats made an average total gain that is intermediate between that made by rats on the diets containing 2% DL-lysine · HCl and either 0.33% or 1.0% of DL-hexahomoserine. The average red blood cell count of the animals receiving inadequate amounts of lysine (Group III) was lower than that of the animals receiving 0.08-0.33% DL-hexahomoserine in the diet.

At autopsy, gross examination of the internal organs of all of the animals was made, and sections of the bone, liver, spleen, testes and kidneys were taken from two or more animals in each group for microscopic examination. No gross abnormalities aside from emaciation were observed among the rats in Groups I-VII inclusive. Five of the rats in Group VIII (including the rat which died on the 24th day) had enlarged spleens. The one animal in Group VIII with a normal spleen had a red blood cell count of 4,900,000 per

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5. Heinemann, W. M., Ensminger, M. E., Cunha, T. J., and McCulloch, E. C., *J. Nutrition,* 1946, v31, 107.

cmm (28th day), was very emaciated (showed no weight gain in 28 days), and apparently was only slightly anemic.

The microscopic sections from the rats selected from Groups I-VI inclusive were negative. The spleen and bone marrow sections from the animals selected from Group VII showed evidence of a small increase in hematopoietic activity. The sections of the spleen and bone marrow from 4 animals with enlarged spleens in Group VIII showed marked hematopoiesis. These animals in Group VIII were either manufacturing new red blood cells at an increased rate, or the new cells were piling up because of the lack of maturation factor(s). The red cell counts made on the blood of the 4 animals with enlarged spleens on the 28th day varied from 3.4-3.9 million per cmm, indicating a definite state of anemia.

The data presented above show that low levels of hexahomoserine can inhibit growth without producing a significant drop in the level of the red blood cells, or a marked change in the blood-forming organs. If the feeding of DL-hexahomoserine at the lower levels had been continued for a longer time than 28 days, it is possible that definite symptoms of anemia may have appeared. Pagé and Gingras(4) have found that the oral administration of DL-hexahomoserine to rats causes a rise in the level of blood plasma amino nitrogen which is not reduced by the addition of lysine to the ration. If the increased plasma amino nitrogen is actually DL-hexahomoserine nitrogen, then the effect of daily ingestion of the compound may be cumulative, and all animals may eventually show growth-retardation even when fed extremely low levels of the compound. Anemia might also occur under these conditions. Studies on the effect of feeding very low levels of hexahomoserine for long periods of time are in progress.

Summary. Young white rats were fed diets containing 2% DL-lysine·HCl and different levels of DL-hexahomoserine for 28 days. When the diet contained 0.08-0.33% DL-hexahomoserine, the growth rate was reduced without simultaneous development of anemia. When the diet contained 1% DL-hexahomoserine, growth was almost completely inhibited and the majority of the animals showed definite symptoms of anemia.

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Cultivation of Mengo Encephalomyelitis Virus in the Embryonated Hen Egg.* (17581)

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Several reports have appeared in the literature on the cultivation of the Columbia SK (1), MM(2,3) and encephalomyocarditis (EMC)(4) viruses. It has been adequately demonstrated(5,6,7) that these 3 agents are closely related to the virus of Mengo encephalomyelitis (ME). It might be expected, therefore, that the pathogenicity of ME virus for the embryonated egg would be similar to that of other agents of this group.

Material and Methods. The strain of ME virus isolated from the central nervous system of a paralyzed rhesus monkey(8) was employed. It had been passed 3 times intracerebrally in mice prior to being used for the inoculation of eggs. The diluent employed in all experiments was a solution of 0.2% bovine albumin in buffered saline(9), containing 200 units of penicillin per ml. The eggs used were of white Leghorn stock incubated 10 to 11 days prior to inoculation. After inoculation, the eggs were incubated at 35°C.

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* This study was made at the Laboratories of the International Health Division of The Rockefeller Foundation, New York, during tenure of a Rockefeller Foundation fellowship, while on leave of absence from the Yellow Fever Research Institute, Entebbe, Uganda.

Inoculation Techniques. Chorio-allantoic membrane and allantoic routes. The methods employed were essentially those of Beveridge and Burnet(10) and Burnet(11) except that in inoculations onto the "dropped" chorio-allantoic membrane, an oval window was cut in the shell. This reduces the danger of damaging the chorio-allantoic membrane. *Amniotic route.* A circular window was cut over the air sac and the shell membrane was clarified with "Bayol" mineral oil. The amniotic sac was then visualized over an egg candler and inoculated directly. This method, which was originally described by Taylor and Saenz (12), gives very low non-specific mortality rates for this route of inoculation. (With the preliminary inoculations by this route, trypan blue was added to the diluent to give a final concentration of 0.025%. By the addition of this dye, it was possible to follow the inoculum in the amniotic sac.) *Yolk sac route.* A procedure similar to that employed for the amniotic inoculations was used, and the yolk sac inoculated directly. The inoculum was 0.2 ml by all routes.

Experimental. Egg passage was initiated by the inoculation of eggs with a 10^{-4} dilution of third mouse brain passage virus. The titer of the virus intracerebrally in mice was $10^{-5.6}$. Groups each of 8 eggs were inoculated by one or the other of the above routes. One hundred percent mortality of all embryos inoculated by the amniotic and yolk sac routes occurred by the third day after inoculation. No deaths of embryos occurred in the eggs inoculated with mouse brain virus by the allantoic route or onto the "dropped" chorio-allantoic membrane during an observation period of 7 days. Using first egg passage virus, the embryo was susceptible to inoculation of virus by all routes. The inoculum was a 10^{-1} dilution of a suspension of whole embryo from the first passage of virus in eggs. (The titer of the virus intracerebrally in mice was $10^{-5.2}$). One hundred percent mortality occurred among embryos inoculated by the amniotic or yolk sac routes by 72 hours of incubation. Of 8 eggs inoculated by the allantoic route with this egg passage virus 2 embryos were dead and 2 were haemorrhagic by the fourth day of incubation, the other 4 embryos remained well. When this same material was inoculated onto the "dropped" chorio-allantoic membrane, all the embryos were dead by 96 hours after inoculation. Egg passage was continued by the amniotic route of inoculation for 7 passages.

Titrations of egg passage virus in mice and eggs. Three dead whole embryos of the first egg passage were removed aseptically, freed of membranes, washed in saline, and ground in a Waring blendor to make a 20% suspension. This suspension was centrifuged at low speed and the supernatant titrated intracerebrally in mice and intra-amniotically in eggs. The LD_{50} for mice was $10^{-5.2}$ and $10^{-5.3}$ for chick embryos. It was found on repeated titration that the end point of infectivity for the chick embryo was as reproducible when egg virus was titrated intra-amniotically, as it is when virus is titrated in mice. By the third egg passage, the titers of virus in whole

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embryo were in the region of 10^{-7}. No significant change in the titer was obtained from the third to seventh passage in eggs.

Rate of multiplication of virus. Two experiments were made to determine the rate of multiplication of virus in chick embryos. In the first experiment, 16 eggs were inoculated by the amniotic route with 84 chick embryo LD_{50} of virus from the unfiltered supernatant of third passage whole embryo suspensions. The embryos of 3 inoculated eggs were harvested, freed of membranes, and pooled 4, 21 and 48 hours after inoculation. The embryos were made into 20% suspensions and stored on dry ice. Subsequently, all suspensions were centrifuged simultaneously in the angle centrifuge. The supernatants were then titrated intracerebrally in mice. In the second experiment, the same inoculum and a similar technique was used, but the embryos were harvested at 24, 48 and 72 hours after inoculation. All embryos harvested up to 48 hours were alive; all, except 1, harvested later were dead. The results of the titrations in these experiments are presented graphically in Fig. 1.

It may be seen that there is a lag in the multiplication of virus up to at least 4 hours after inoculation. Multiplication of virus then takes place rapidly up to 48 hours. After that time there is no significant change in the titer of virus in the embryo up to the time of death of the embryos.

Rate of multiplication of virus after inoculation by various routes. Groups, each of 12 eggs, were inoculated by amniotic, allantoic, chorio-allantoic, and yolk sacs and allantoic fluids were harvested separately after 48 hours of incubation. The embryos and yolk sacs were allowed to drain and were pooled and then ground up as 20% suspensions in a Waring blendor. Dilutions of the supernatants of these suspensions and of a pool of the allantoic fluids were titrated intracerebrally in mice. The results of the titrations of the whole embryos and yolk sacs are presented in Table I.

High titers of virus were present in the embryo and yolk sac 48 hours after inoculation by all routes except the allantoic route. The titer of the allantoic fluid irrespective of the route of inoculation was not found to be greater than $10^{-1.7}$ up to 48 hours of incubation.

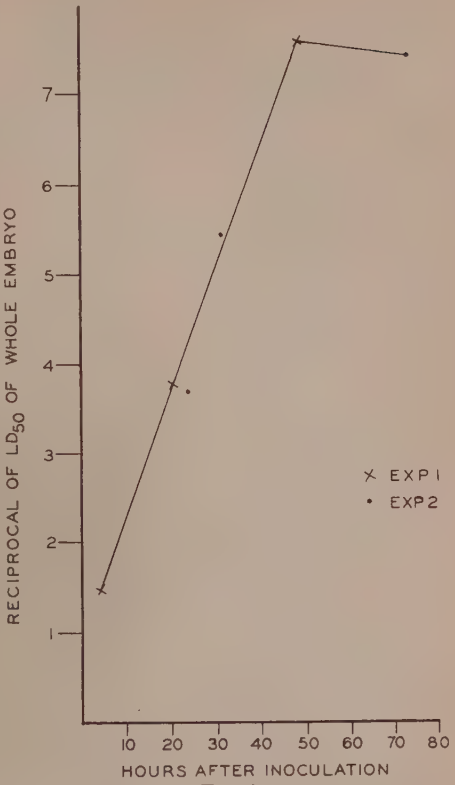

FIG. 1.

Rate of multiplication of ME virus in chick embryo after amniotic inoculation.

Pathology of infected embryos.† Dead embryos were very congested and showed multiple haemorrhages. Sections of these embryos showed widespread degenerative changes and haemorrhages were present throughout the entire embryo. In embryos harvested at 24 and 48 hours after inoculation, no macroscopic changes were observed, except occasional haemorrhagic areas. Sections of these embryos showed foci of degenerative changes in the skin, musculature and visceral organs. No specific lesions were found in the central

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† I am indebted to Dr. H. N. Johnson, staff member of the International Health Division, The Rockefeller Foundation, for the preparation of the histological material.

TABLE I.
Titrations in Mice of Whole Embryos and Yolk Sacs of Eggs Inoculated with Mengo Encephalomyelitis Virus by Various Routes.

Tissue tested	Route of inoculation	Reciprocal of log of LD50 of tissue harvested at 48 hr
Embryo	Amniotic	7.6
	Yolk sac	5.9
	C.A. membrane	4.8
	Allantoic	<1.5
Yolk sac	Amniotic	6.3 or >
	Yolk sac	6.3 or >
	C.A. membrane	4.8
	Allantoic	<1.5

C.A. = Chorio-allantoic.

nervous system. The chorio-allantoic membranes of embryos inoculated by that route were edematous, and opaque and sections showed proliferative and degenerative changes.

Discussion. Warren(4) reported that EMC virus causes death of embryos within 72 and 96 hours after inoculation and that emulsions of infected embryos were infectious at dilutions of 10^{-7} when titrated intracerebrally in mice. Allantoic fluids of eggs inoculated with EMC virus are said to have titers of 10^{-4} to 10^{-5}. Schultz *et al.*(1) showed that Columbia SK virus was readily propagated in fertile eggs, by the yolk sac, chorio-allantoic and "intra-embryonic" routes and that deaths of embryos occurred from the first passage. The titration of filtrates of whole embryos from the 15th to 30th passage was 10^{-7}. The behavior of MM virus was similar to that of Columbia SK virus(2).

Like the other agents of this family of viruses, ME virus was very readily adapted to the chick embryo. The virus may be as readily titrated by amniotic inoculation of eggs as in mice. The titer of embryos of first egg passage virus was 10^{-5} and by the third passage titers in the region of 10^{-7} were obtained in whole embryos harvested 72 hours after inoculation. The chick embryo yielded the highest titers after amniotic or yolk sac inoculation. The titer of virus in the allantoic fluid was found to be less than 10^{-2} irrespective of the route of inoculation when the fluids were harvested 48 hours after inoculation. It is possible that higher titers of virus might be obtained in the allantoic fluid at 72 hours after inoculation, at which time the titer in whole embryo is at its maximum. This low titer of virus in the allantoic fluid is in keeping with the findings of Powell and Jamieson(3) who reported that with MM virus, the "periembryonic fluids" gave titers in mice of less than 10^{-2}.

The rate of multiplication of ME virus is similar to that which has been observed in the cultivation of the equine group of virus encephalitides in eggs.

Summary. Mengo encephalomyelitis (ME) virus was readily propagated in the embryonated hen egg. Infected embryos showed extensive haemorrhages and were usually dead within 48 to 96 hours after inoculation. Sections showed widespread degenerative changes and haemorrhages throughout the entire embryo. The embryo was susceptible to inoculation of ME virus by the amniotic, yolk sac, and allantoic routes and onto the "dropped" chorio-allantoic membrane. After amniotic inoculation, there was a slight lag in virus multiplication, after which rapid multiplication occurred and titers of virus of 10^{-7} were obtained in suspensions of whole embryos by 48 hours after inoculation. Similar titers were obtained in suspensions of whole embryos and yolk sacs after inoculation by the amniotic or yolk sac routes. The embryo was less susceptible to inoculations by the allantoic route. Irrespective of the route of inoculation, the titer of virus in the allantoic fluids was always less than 10^{-2} up to 48 hours after inoculation. The behavior of ME virus in the embryonated hen egg was similar to what has been described after inoculation of eggs with Columbia SK, MM and encephalomyocarditis viruses.

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Effect of Desoxycorticosterone Acetate on Insulin Sensitivity in Diabetes Mellitus.* (17582)

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It has been shown by Cheng and Sayers(1) that desoxycorticosterone acetate (DOCA) increases the sensitivity of rats to insulin, presumably through the previously demonstrated ability of adrenal cortical hormones to inhibit the pituitary output of ACTH.(2) The demonstration of a similar effect of DOCA should theoretically be possible in the type of human diabetes mellitus where the activity of a pituitary factor appears to play a role. Himsworth(3) has suggested that this type of diabetes is present when the glucose-insulin tolerance curve indicates a relative insensitivity to insulin, in contrast to the variety of diabetes in which the glucose-insulin tolerance curve shows a sensitivity to insulin. The present study was undertaken to determine whether insulin insensitive type of curves, as observed in certain patients with diabetes mellitus, can be converted to insulin sensitive type.

Methods and Procedures. Six adult diabetics found to be insulin insensitive by the Himsworth glucose-insulin tolerance test(3) were given 10 mg of DOCA, intramuscularly, daily. In 5 of these patients the injections were continued for 10 days, in one for 4 days. The glucose-insulin tolerance and glucose tolerance tests were done consecutively on successive days before the administration of DOCA and directly after its discontinuance.

Results. In this report only the effects on the glucose-insulin tolerance test are included. The effects of the administration of DOCA on the glucose tolerance, glucose excretion in the urine, fasting blood sugar and insulin requirements will be reported at a later date. From Fig. 1 it can be seen that 5 of the 6 patients showed greater degree of sensitivity to insulin after receiving DOCA than they had before. The curves, which before treatment were characteristic of insulin resistance, changed to those typical of insulin sensitivity. The curves of the sixth patient (G. E.) showed minimal changes, which were in the direction of increased insulin sensitivity, but not beyond the range of spontaneous variability.

Comment. The data presented here seem to show that administration of DOCA to insulin insensitive diabetic patients is followed by increased sensitivity to insulin. This par-

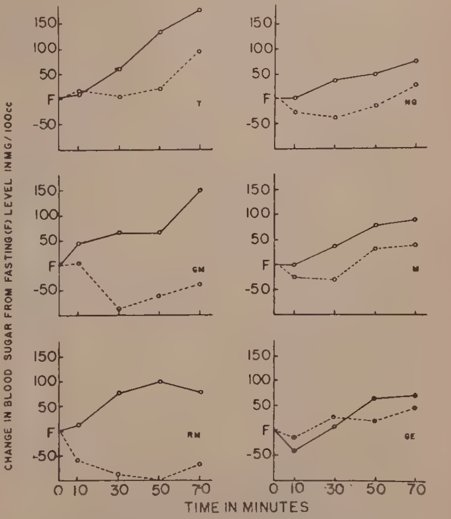

FIG. I EFFECT OF CONTINUED ADMINISTRATION OF DOCA ON THE GLUCOSE-INSULIN TOLERANCE TEST

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* Sponsored by the VA and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are a result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

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allels the observation of Cheng and Sayers(1) in non-diabetic rats. These authors suggest that the mechanism of this change is most likely through inhibition of the pituitary output of diabetogenic factors, although direct competition with 11-oxy-steroids must also be considered. The estimation of the changes in output of 11-oxy-steroids by diabetics receiving DOCA will throw additional light on this problem. Further observations on the dynamics of this reaction in humans are in progress.

Summary. The effect of DOCA on the insulin sensitivity of 6 insulin insensitive patients was studied. Five showed a marked increase, and one an equivocal increase in insulin sensitivity.

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Production of Hypertension in the Rat by Substituting Hypertonic Sodium Chloride Solutions for Drinking Water.* (17583)

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A disturbance in the metabolism and distribution of salt and water in animals made hypertensive by the Goldblatt technique and its modifications and in essential hypertension may be inferred from a considerable body of recent work. An increase in the volume of extracellular fluid has been demonstrated in the muscles of hypertensive dogs;(1) polyuria and polydipsia are noted in renally hypertensive rats;(2) increased excretion of antidiuretic hormone has been reported in hypertensive dogs, rats and men;(3) the movements of extracellular fluids in rats made hypertensive by subtotal nephrectomy in response to the administration of hypertonic solutions are altered from the normal.(4) In free choice experiments hypertensive rats voluntarily reduce their sodium chloride intakes,(5) and rigid sodium chloride restriction lowers the blood pressures of hypertensive rats.(6) The utilization of low sodium diets in the therapy of hypertensive disease in man has been reviewed recently,(7) and the hypertensive patient reacts to the withdrawal of dietary sodium chloride in a manner different from the normal.(8) It appears possible, from a consideration of the known effects of adrenal steroids on the extracellular fluid, that the hypertension resulting from the administration of desoxycorticosterone acetate and sodium chloride(9) may also be associated with a disturbance in the extracellular fluids. Although it was originally believed that this type of hypertension might be explained by the renal lesion resulting from the procedure,(10) this view has become untenable in the light of recent evidence which indicates that such

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† Senior Assistant Surgeon (R), United States Public Health Service, on special assignment from the National Institutes of Health.

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hypertension may persist in the absence of the kidneys(11) and that it is promptly reversible on the removal of DOCA implants.(12) Furthermore, the rapid disappearance of hypertension in animals with Goldblatt hypertension subjected to bilateral adrenalectomy,(13, 14,15) a phenomenon for which adequate explanation is still lacking, may be associated with the effects of adrenalectomy on the extracellular fluid.

The possibility that there may be an etiological relationship between a disturbance in the extracellular fluid and hypertension has recently been emphasized by Covian and Braun-Menendez(16) who found that after bilateral nephrectomy certain rats developed hypertension, and that the appearance of this hypertension appears to be related to an increase in the volume of the extracellular fluid whether this occurs spontaneously or is produced by the administration of sodium chloride solutions. The possibility that disturbance of the extracellular fluid may be of etiological significance in hypertension has been the subject of investigations in this laboratory. In the present report experiments are described which were designed to test the hypothesis that the extracellular fluid disturbance might be duplicated and hypertension induced by presenting animals with solutions of sodium chloride, in *concentrations sufficient to tax the regulatory functions of the kidney,* as a substitute for the drinking water. Although it has not proved possible to induce or to aggravate hypertension in the rat or in man by the administration of large quantities of sodium chloride,(17,18,19) the important factors of controlled water intake and sufficient prolongation of the experiment have not been achieved in any experiments on mammals. Lenel, Rodbard and Katz(20) have produced hypertension in the chicken by the substitution of isotonic and slightly hypertonic solutions for the drinking water, but similar results have not been reported for the mammal.

Experimental. Rats of the USC strain were used in these experiments. In 3 experiments adult males, young females and adult females were used. The animals of Experiments I and III weighed 200-250 g at the start of the experiment, those of Experiment II weighed 100 to 125 g. After a preliminary period in which body weights, fluid intakes and blood pressures were determined, the animals were divided into control and experimental groups. Blood pressures were determined with the aid of the Sobin tail plethysmograph.(21) The experimental animals received sodium chloride solutions from graduated drinking bottles as their sole source of fluid while control animals received tap water. In Experiment I sodium chloride concentrations of 1.5 to 2.5% were given; in Experiment III 2.0% sodium chloride solutions were used throughout; in Experiment II, which was conducted in the hottest part of the summer, the animals received 2.0% sodium chloride through most of the experimental period, but refusal of the salt solutions and precipitous weight losses on very hot days necessitated the substitution of tap water for 24 hour periods on several occasions. For the most part, salt concentrations of 2.0% were well tolerated by the animals; edema was never observed and diarrhea occurred only occasionally.

Blood pressures, body weights, and fluid intakes were followed for 6 weeks after the institution of the hypertonic sodium chloride regime. At the end of this period the animals

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were sacrificed and the weights of hearts and kidneys determined. The results of the experiments are presented in the Table. Blood pressure measurements obtained in the early part of the experiment indicated that the rise of blood pressure occurred rather suddenly in any rat after a latent period of one to 4 weeks. Once hypertension appeared in any animal it persisted so long as the animal continued on the regime of hypertonic saline. Although the pressure readings in any animal showed considerable variability when hypertension was established, normotensive readings were obtained only infrequently after the initial appearance of hypertension. No definite trend toward aggravation of the hypertension could be discerned in examination of the protocols of individual animals in the period of the experiment although the average of the systolic pressures of the "salt" groups showed a tendency toward persistent rise as increasing numbers of animals became hypertensive.

At autopsy the experimental animals showed no gross abnormalities beyond the enlargement of the heart and kidneys relative to the body weight noted in the Table. Although it will be noted that the absolute weights of these organs do not show significant increases, we feel that we are justified in considering that a true cardiac hypertrophy has developed in view of the constancy of the relationship between heart weight and body weight in rats subjected to inanition.(22)

Discussion. From our results it is clear that arterial hypertension can be produced in the rat by the substitution of hypertonic saline solutions for the drinking water. It is our belief that the present results, taken in conjunction with some earlier findings in which one of us (LAS) was unable to produce hypertension by the continued administration of large quantities of isotonic salt solutions, and the work of others (see above) who have failed to produce hypertension by the administration of salt without controlling water intake, indicate that the *ratio of sodium chloride to water intake rather than the amount of sodium chloride taken is the determining factor* in the

TABLE I.

Systolic Blood Pressures and Organ Weights of Rats Receiving Hypertonic Sodium Chloride Solutions as Substitute for Drinking Water.

Exp. No.	Treatment	No., sex of rats surviving 4-6 wk	Body wt terminal (g)	Fluid intake,* cc/rat/day	Systolic blood pressure* (mm Hg)	Heart wt × 1000 / Body wt	Kidney wt × 1000 / Body wt
I	Control	7 M	250	23	103 ± 2	3.04 ± .22	7.1 ± .52
	1.5-2.5% NaCl	12 M	216	61	136 ± 20	3.43 ± .31	9.0 ± .85
II	Control	10 F	179	50	101 ± 4	3.07 ± .22	8.4 ± .69
	2% NaCl (see text)	10 F	141	196	122 ± 13	3.99 ± .32	9.8 ± .95
III	Control	10 F	237	44	99 ± 6	3.09 ± .27	7.6 ± .28
	2% NaCl	9 F	165	111	138 ± 7	4.16 ± .45	11.4 ± 1.51

* All values represent means in the last two weeks of the experiment.

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development of this type of hypertension. Further study is required to elucidate the mechanism of this hypertension. One possibility which suggests itself is that the hypertension is due to the synergism between large quantities of salt and the endogenous secretions of the adrenal cortex. We are inclined to doubt this view, for work now in progress indicates that "salt" hypertension is not abolished by bilateral adrenalectomy, in contradistinction to hypertension of the Goldblatt type. Another possible interpretation is that the development of renal hypertrophy may result in a relative renal ischemia with the evocation of hypertension of the Goldblatt type. We consider this view unlikely, for we have noted no significant lesions in the kidneys of our hypertensive animals beyond the hypertrophy.

Although, as noted above, there is considerable evidence that arterial hypertension of several types is associated with a disturbance in extracellular fluid, an exact characterization of the changes in the extracellular fluid in hypertension has not been made, nor are we at present in a position to state the nature of the changes in the body fluids which occur in animals watered with hypertonic saline solutions. Further investigations in these directions will be required before it will be possible to attribute any etiological significance to body fluid alterations in hypertension.

Summary and Conclusions. (1) In 3 experiments, systolic blood pressure measurements were made on 27 control rats and on 31 animals watered with hypertonic sodium chloride solutions for a period of 6 weeks.

(2) The animals watered with hypertonic saline solutions developed an arterial hypertension after a latent period of one to 4 weeks. At autopsy this was found to be associated with an hypertrophy of the heart and kidneys relative to body weight.

(3) Some possible mechanisms for the development of this hypertension are discussed.

(4) The substitution of hypertonic sodium chloride solutions for the drinking water affords a simple, inexpensive, and dependable method for the production of arterial hypertension in the rat.

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Relation Between Pantothenic Acid and Response to Growth Hormone in the Adult Rat. (17584)

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The experiment reported here was originally designed to test the response of rats on high fat and high carbohydrate diets to anterior pituitary growth hormone. During the course of the experiment it was discovered that the diets were deficient in pantothenic acid. This circumstance, when coupled with the injection of growth hormone, produced results that seemed sufficiently interesting to report.

Methods. Adult female rats of the Sprague-Dawley strain were used in this study. There were 7 rats in each group. One group was placed on a high fat, and the other group on a high carbohydrate diet. Each high fat animal was pair fed with a high carbohydrate animal of similar body weight. Differences in caloric value of equivalent amounts of the fat and carbohydrate sources were taken into account in the paired feeding in order to insure isocaloric intake between the animals of each pair. Both diets contained 20% protein as casein, inorganic salts, Wesson oil, cod liver oil, tocopherols, and B vitamins in excess. The high fat diet contained 37% Crisco and the high carbohydrate diet 74% sucrose. The rats were allowed a preliminary

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The Effect of Pantothenic Acid Deficiency on the Response to Pituitary Growth Hormone.

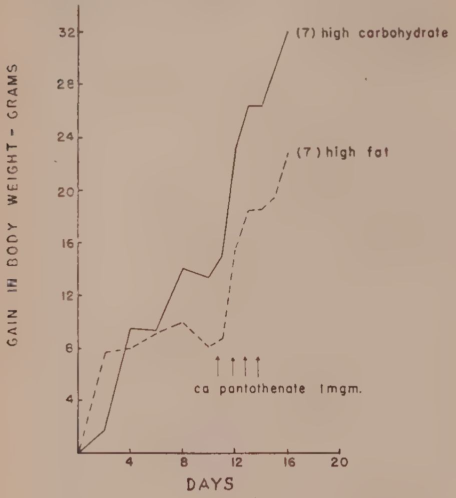

FIG. 1.

An experiment illustrating the essential nature of pantothenic acid in the body weight response of adult female rats to growth hormone. See text for details.

period of 2 weeks on these diets in order to become accustomed to them. During this period it was established that the weight curves of both groups had "plateaued," and that both groups were maintaining approximately equal weights. There then followed an experimental period of 16 days during which each rat received a daily intraperitoneal injection of 1.0 mg of a crystalline anterior pituitary growth hormone.† Body weight was measured daily and increments over the control weights were determined.

Results. In Fig. 1 are presented the results of the experiment. Each curve represents the average weight gain over the control value, for the group. The solid line represents the high carbohydrate group, and the dashed line the high fat group. During the first week of the experiment the animals of both groups showed a definite gain in weight in response to the growth hormone administration. However, in the second week it was noted that both groups were not gaining as rapidly as before and were not completely consuming their ration. Indeed they started to lose weight and show signs of a deficiency of one of the B vitamins. There was porphyrin encrustation of the whiskers and fur on the top of the head, red painful feet, bloody encrustation about the external nares, and general loss of luster and matting of the coat. These signs were much more marked in the animals on the high fat diet. It is to be noted that their weight curve dropped below that of their high carbohydrate pairs. An examination of the diet at this time revealed that pantothenic acid had been omitted. Accordingly each rat was given an intraperitoneal injection of 1.0 mg of calcium pantothenate on the eleventh day and for 3 days thereafter. It is evident from the figure that both groups showed a dramatic increase in weight gain after this vitamin. This specific response, coupled with a complete disappearance of the other clinical signs of the deficiency, would seem to establish the existence of a true pantothenic acid deficiency.

Discussion. It should be emphasized that the results presented herein are those of a single experiment. Nevertheless they indicate that, under the circumstances of this experiment, a deficiency of pantothenic acid was produced in adult female rats whose weight curves had "plateaued". Under ordinary conditions a deficiency of pantothenic acid is difficult, if not impossible, to produce in an adult rat. Apparently when such an adult animal is induced to grow again at a rapid rate, as after the injection of growth hormone, the requirements for pantothenic acid are sufficiently increased to produce signs of a deficiency of that vitamin if sufficient supplies are not available. Furthermore if the animal is on a high fat diet under these conditions the requirements for pantothenic acid are still greater, and hence the degree of deficiency is more severe. The reasons for this effect of fat are not immediately obvious; however they might be explained on the basis of the carefully performed experiments of Forbes and his associates.(3) These workers studied the relative efficiency, in terms of nitrogen retention, of high fat, as versus low fat diets. They found that the diet high in fat caused the retention of more nitrogen than the low fat diet, when the protein intake was the same in both instances. Thus the energy made available by the combustion of fat caused more protein to be synthesized than the energy from an equicaloric quantity of another source. Therefore the animals on a high fat diet, who were receiving growth hormone at the same time, were getting a double stimulus to their protein synthetic process. This would put a greater strain on their reserves of pantothenic acid than that experienced by the animals on the high carbohydrate diet, and thus produce a more severe deficiency.

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Lipmann and his associates(1,2) have clearly demonstrated that pantothenic acid exists in the tissues in the form of a coenzyme that is vitally concerned in acetylation reactions. They have studied these reactions extensively in the case of the acetylation of sulfanilamide in the liver. They believe that in the process of acetylation there is an intermediate stage in which the carboxyl group is phosphorylated, and thus "activated", as a preliminary to condensation. Since this process of acetylation of sulfanilamide represents the synthesis of a peptide bond, and since coenzyme A is necessary for its normal occurrence, it is logical to assume that pantothenic acid, in the form of this coenzyme, is involved in the reactions leading to the synthesis of proteins. Under conditions where there is an exaggerated rate of protein synthesis, as after the administration of the growth hormone, the rate of synthesis is depressed if adequate amounts of pantothenic acid are not available.

Summary. It has been shown that the adult female rat, whose growth curve has "plateaued", can be made to develop an acute pantothenic acid deficiency when stimulated to grow rapidly by the continued injection of anterior pituitary growth hormone. The deficiency, so produced, develops more rapidly and is more severe if the deficient diet is high in fat. It is concluded that pantothenic acid is required for the synthesis of protein in the body.

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Use of Radioactive Phosphorus to Determine Volume of Blood Replaced in Replacement Transfusion. (17585)

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Replacement transfusion has been advocated recently in the treatment of acute leukemia and acute renal failure.(1-3). The volume of blood replaced by such a procedure may be calculated mathematically if the mixing of blood in the body behaves as it would in a simple mechanical system. The agreement of a determination (using differential Rh typing) of the volume of blood replaced in a case of erythroblastosis fetalis(4) with a mathematically derived value supports this concept. Since the technic of labeling erythrocytes with radioactive phosphorus(5) provides a simple and widely applicable method for the determination of blood volumes, it was decided to evaluate the usefulness of this technic in the

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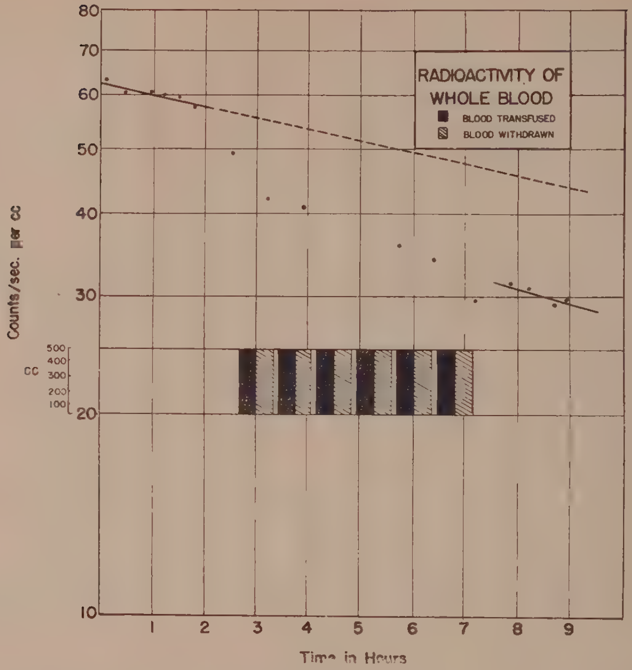

FIG. 1.
Graphic expression of data obtained from subject 2 (Table 1). The solid lines are drawn through the pretransfusion points and the post transfusion points. The interrupted line is extended from the solid line drawn through the pretransfusion points for the determination of predicted activity.

measurement of the volume of blood replaced in replacement transfusions, comparing the experimental results with values calculated mathematically.

Methods. Four replacement transfusions were carried out in 3 patients with acute leukemia and in one patient with chronic leukemia. Ten to 15 ml of whole blood were withdrawn in a heparinized syringe from the patient. The blood was incubated under sterile conditions with 100 microcuries of P^{32} at 37°C for a period of 2 hours with gentle stirring every 15 minutes. Following incubation, the blood was centrifuged; the sedimented cells were washed twice with normal physiological saline and resuspended to the original volume with saline. An aliquot of the resuspended cells was reinjected intravenously into the patient and another aliquot saved for radioactive assay. At 15, 30, 60, 90 and 120 minute intervals after the injection, samples of heparinized blood were withdrawn for radioactivity assay. After this 2 hour equilibration period, the replacement transfusion was carried out either by simultaneous continuous transfusion and withdrawal of blood, or by intermittent transfusions and withdrawals (500 ml at a time). The amount of blood transfused was varied from 1,075 ml to 12,500 ml. At the completion of the replacement transfusion, blood was again withdrawn after 15, 30, 60, and 120 minutes for radioactivity assay. For determination of radioactivity 0.050 or 0.075 ml of whole blood was plated on copper planchets and counted with a mica window Geiger Muller tube. Counts were 4 to 20 times background.

Determination of volume of blood replaced

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TABLE I.
Comparison of Determined and Theoretical Amounts of Blood Replaced.

Subject	Amt of blood transfused and withdrawn (ml)	Predicted activity (cps/ml)	Determined activity (cps/ml)	Determined percentage of original blood replaced (%)	Theoretical percentage of original blood replaced (%)	Difference (%)
1	1075	114	93.2	18.3	19.3	—1.0
2	3000	46.5	29.5	36.5	37.4	—0.9
3	3000	41.0	26.0	36.5	40.4	—3.9
4	12500	150	11.5	92.4	96.3	—3.9

using experimental data. The radioactivity of the blood was expressed as counts per second per ml and was plotted logarithmically as a function of time. Straight line curves were drawn through the pretransfusion points and the post transfusion points. The radioactivity, expressed as counts per second (cps) per ml, at any given time after the replacement transfusion was designated as *determined activity.* The radioactivity of the blood at the same given time, had no transfusion been given, was then estimated and designated as *predicted activity.* The predicted activity reflects the loss of P^{32} from the erythrocytes by exchange with plasma phosphorus. It was determined by extrapolating the pretransfusion curve to the time chosen for the determined activity. (Fig. 1). The percentage of the original blood replaced was calculated by the following formula:

$$\text{Percentage of blood replaced} = 100 - \frac{\text{determined activity (cps/ml)}}{\text{predicted activity (cps/ml)}} \times 100.$$

This calculation is based on the assumption that the rate of phosphorus exchange remains constant throughout the experimental period. This assumption is borne out by the similarity of slopes of the curves before and after transfusions.

Determination of the volume of blood replaced using mathematical formulae. If the whole blood volume is known, the percentage replacement may be calculated according to the following formulae:

a. $\frac{N}{n} \log_e n = C$

b. Percentage of blood replaced =

$$\left[1 - \frac{1}{n} \cdot e^{\left(\frac{-n(t-c)}{N}\right)}\right] \cdot 100$$

Where: n = volume of blood transfused; t = number of transfusions; N = whole blood volume calculated according to the method of Nieset, *et al.*(5)

Results. The percentages of blood replaced found by using the experimental data (determined %) and the mathematical formulae (theoretical %) are given in Table I. A comparison of the values arrived at by the two methods reveals a difference in percent ranging from 0.9 to 3.9.

Summary. Using erythrocytes tagged with radioactive phosphorus, the percentage replacement of blood in replacement transfusion can be determined. The values obtained agree well with mathematically derived values.

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Use of Complement Fixation in the Differentiation of Strains of Coxsackie Virus. (17586)

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During the past year a group of viruses has been isolated from the tissues and excretions of individuals suffering from illnesses of undetermined etiology. These agents are lethal for suckling mice and hamsters and are similar to the recently designated Coxsackie viruses isolated and described first by Dalldorf and Sickles, *et al.*(1,2) and later by Melnick, Shaw, and Curnen(3). It had been observed by these workers and also in this study that the Coxsackie virus strains were not all serologically similar. After receiving antisera for viruses designated as Types 1, 2, and 3 from Dr. G. Dalldorf of the New York State Laboratory, it was found by means of the neutralization test that certain of the strains isolated here corresponded to Types 1 and 2, but none so far to Type 3. Two additional distinct types have been differentiated in this laboratory and are called Types 4 and 5. Although the strains obtained could be typed by the neutralization method, it was thought advisable to determine whether the complement fixation test could be used both for this purpose and for determining the presence of antibodies in human sera.

Methods. (a) *Immune Sera.* Ten per cent suspensions of mouse brains or filtered muscles containing active virus for each of Types 1, 2, 4, and 5 were prepared in buffered saline and kept frozen until needed. Six-week-old mice were given biweekly intraperitoneal injections of 0.2 ml of the stock vaccines over a period of 3 weeks. One week after the last inoculation, the animals were bled and the serum frozen. Adult hamsters could be immunized in the same manner with infected hamster tissues.

(b) *Antigens.* Filtered muscle tissue was used for the antigens because the skeletal muscles of suckling mice or hamsters contained high concentrations of virus. The whole mouse could be employed also after removal of viscera, but did not seem quite as potent as the more concentrated muscle virus filtrates. The skeletal muscles were removed from 20 to 30 mice that had shown definite muscle paralysis from 2 to 5 days after intramuscular inoculation of 0.03 ml of virus. The skinned muscles were weighed and ground in a Waring blendor with buffered saline to make a 20% suspension. The material was removed to a flask, left overnight at 4°C, and then centrifugated for 30 minutes at 2500 rpm in the horizontal centrifuge. The supernatant fluid was passed through a Seitz filter. After alternately freezing and thawing the filtrate 5 times in an alcohol-dry ice mixture, it was centrifugated for ninety minutes at 13000 rpm in the angle centrifuge in the cold room. The supernatant fluid was stored in small bottles at 4°C. No preservative was added. The antigens for each type of virus were standardized, and usually 4 units of antigen were used in the test against human sera. However, the antigen unitage was not determined when typing new strains since no homologous antiserum was available; therefore, undiluted antigen for each new virus was used against standard antisera of the known types.

(c) *The Complement Fixation Test.* The complement fixation tests were performed according to a modified Kolmer technic employing one-half the quantities with a total volume of 1.5 ml. Three per cent washed sheep red blood cells and 2 full units of complement were used, the complement having been titrated with the antigens before each test. Serum, antigen, and complement were placed in the

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* From the Virus and Rickettsia Branch, Laboratory Division, Montgomery, Ala.

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TABLE I.

Comparison of Complement Fixation and Neutralization Tests in Typing Coxsackie Viruses.

Location of viruses isolated	Total cases typed	No. pos. by C.F. Virus type 1	2	4	5	No. pos. by neutralization Virus type 1	2	4	5
Alabama	13	1	2	8	2	1	2	8	2
Delaware	4	1	1	2		1	1	2	
Florida	2				2				2
Georgia	1		1				1		
Louisiana	4		3	1			3	1	
Oklahoma	1		1				1		
Tennessee	10	1	3	5	1	1	3	5	1
Totals	35	3	11	16	5	3	11	16	5

TABLE II.

Comparison of Neutralization and Complement Fixation Tests with Coxsackie Virus Antigens.

Patient's sera	A. Neutralization* No. days after onset	Coxsackie viruses Type 1	Type 2	B. Complement fixation† Type 1 antigen Serum dilutions 1/2	1/4	1/8	1/16	Type 2 antigen Serum dilutions 1/2	1/4	1/8	1/16
B.	2	+	+	2	0	0	0	4	4	3	0
	35	+	+	4	2	1	0	4	2	1	0
C.	5	0	+	0	0	0	0	4	4	4	1
	34	0	+	0	0	0	0	4	4	1	0
D.C.	4	0	0	0	0	0	0	4	4	4	4
	28	0	+	0	0	0	0	4	4	4	4
L.C.	1	0	0	0	0	0	0	0	0	0	0
	18	0	0	0	0	0	0	0	0	0	0
J.	10	0	0	3	0	0	0	4	1	0	0
	42	0	+	4	0	0	0	4	4	4	1
H.	13	+	+	4	4	0	0	0	0	0	0
	44	+	+	4	3	0	0	0	0	0	0
K.	4	+	+	3	1	0	0	4	2	0	0
	26	+	+	4	4	2	0	4	4	3	0
P.	1	0	0	0	0	0	0	0	0	0	0
	26	+	0	4	4	4	—	0	0	0	0

* + = Sera neutralized by at least 1000 LD50 doses of virus. All sera inactivated at 58°C for ½ hr.

0 = Sera did not neutralize 1000 LD50 doses of virus.

† Figures refer to degree of hemolysis: 0 = complete hemolysis; 1, 2, 3 = intermediate hemolysis; 4 = no hemolysis.

refrigerator overnight at 4°C before adding the sensitized red cells. After incubation for thirty minutes at 37°C in the water bath, followed by one hour in the refrigerator, the results were recorded at 4+ (no hemolysis), 3+, 2+, 1+ (intermediate hemolysis), and 0 (complete hemolysis). Controls included those for the sera, antigens, hemolytic system, and known positive sera from each type. Three and four plus reactions were considered of significance in serum dilutions above 1-2, but equivocal if in the latter dilution.

Results of the Tests. The strains of Coxsackie virus could be separated easily into different types by means of the complement fixation method, and in each case there was agreement with the results of the neutralization tests (Table I). A total of 35 strains has been tested with the majority falling into Types 2 and 4. None so far has corresponded to Type 3 of Dalldorf.(2) A later report will be made when the tests for all of the strains on hand have been completed.(4) The results with the human sera were not generally so clear cut. Twenty-two pairs of acute and convalescent sera were tested against antigens of Types 1 and 2. Typical results for 8 pairs are given in Table II. An occasional rise in titer for the same strain was noted in both tests. Often the antibodies were present soon after the onset. In general, there was agreement between the two methods as to the antibody types found. If the neutralization test was positive for Type 1, for instance, the complement fixation test was positive for the same type. Occasionally antibodies were found for both types in each test.

Summary. The complement fixation test using filtered muscle antigen can be employed for differentiation of the Coxsackie virus into serologically distinct types and for determining the presence of antibodies in human sera.†

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† Since submitting this paper for publication, Casals, Olitsky and Murphy also have reported on the use of the complement fixation test for differentiating the Coxsackie viruses. PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 636.

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A Short Column Procedure for Separating Cytoplasmic Components from Normal and Tumor Tissue. (17587)

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Columnar chromatography has been shown to be applicable to purification and characterization studies of certain subcellular particulates of wide size and physiological variation, namely the virus-like agent of chicken tumor I(1) and the microscopically visible granules of the Harding-Passey and the S91 mouse melanomas.(2) Most of these studies were made with material that had already been partially purified by differential centrifugation. It has since been found, in working with the latter tissues, that the preliminary purification can be done rapidly without recourse to the centrifuge by employing a procedure involving grinding of the tissue with diatomaceous silica (Celite No. 503) and submitting this slurry to a short column or pad of the same adsorbent. The resulting percolate consists essentially of the subcellular granules and most of the soluble components of the tissue extract. It is devoid of nuclei and intact cells as indicated by microscopic examination. This tissue percolate is suitable for certain enzyme investigations or further purification studies by chromatography or differential centrifugation.

Methods and results. A typical procedure is outlined in the following experiment indicating the relative results obtained with equal quantities of tumor tissue prepared by the short column technic as compared with standard grinding followed by centrifugation. Several Harding-Passey tumors were pooled and fragmented by forcing through a 5 ml syringe without needle, and divided into 2 equal lots of 5 g each termed A and B. Lot A was ground in an iced mortar without sand and with the gradual addition of 5 times its weight of cold distilled water. This suspension was then cleared of tissue debris, unbroken cells, and nuclei by two successive centrifugations† of 5 minutes each at approxi-

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* With the aid of John Chambers.

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† Type SS-1 Serval centrifuge with 9″ angle rotor, run at approximately 3700 rpm in a walk-in refrigerator at 5°C.

TABLE I.
Comparison of Harding-Passey Fractions Prepared by Centrifugation and the Short Column Procedure.

Criteria	Extract (A) Centrifuge	Extract (B) Short column	Resuspended pellets (A) Centrifuge	Resuspended pellets (B) Short column
Grams of tumor	5.0	5.0	—	—
Vol. of extract, ml	50.0	50.0	—	—
Optical density (× 10) 650 mu	10.6	13.0	9.2	11.5
Dry wt, mg/ml	10.4	11.6	4.2	4.5
Nitrogen, mg/ml*	1.01	1.14	0.41	0.42
Phosphorus, mg/ml*	0.10	0.12	0.03	0.03
Succinoxidase activity†				
$\mu l\ O_2/hr$	237.5	248.6	159.0	167.9
QO_2	22.8	21.4	37.9	37.3
$QO_2(N)$	235.1	218.1	387.8	399.8
Dopa oxidase activity‡				
$\mu l\ O_2/hr$	287.6	332.0	100.0	102.4
QO_2	27.7	28.6	23.8	22.8
$QO_2(N)$	284.8	291.2	243.9	243.8

* Determinations made in the NIH micro-chemical laboratory under the supervision of Mr. William C. Alford.

† Succinoxidase activity was determined manometrically with the following vessel contents: 1.0 ml of tissue fraction, 0.2 ml of succinate-cytochrome C mix composed of 1/3 commercial cytochrome C (VioBin) and 2/3 sodium succinate, 0.5 M; 0.1 ml 2N KOH in center well. All experiments were run at 38°C. The values were calculated from the first 30 minutes uptake.

‡ Dopa oxidase activity was determined as above except 1 mg of dopa in 0.2 ml distilled water was substituted for succinate-cytochrome C in the sidearm.

TABLE II.
Comparison of S91AA Amelanotic Tumor Fractions Prepared by Centrifugation and by the Short Column Procedure.

Criteria	Extract (A) Centrifuge	Extract (B) Short column	Resuspended pellets (A) Centrifuge	Resuspended pellets (B) Short column
Grams of tumor	5.0	5.0	—	—
Vol. of extract, ml	50.0	50.0	—	—
Nitrogen, ml	0.91	1.18	0.18	0.23
Succinoxidase activity				
$\mu l\ O_2/hr$	148	167	69	88
$QO_2(N)$	162	141	372	374
Cytochrome oxidase activity*				
$\mu l\ O_2/hr$	240	330	266	277
$QO_2(N)$	262	280	1440	1180

* Determined in the same way as succinoxidase activity except that PPDA was substituted for succinate at a vessel concentration of 0.02 molar.

mately 1500 g.(3) The supernatant fluid was diluted with cold distilled water to 50 ml and called extract A.

Lot B was ground in a mortar in the same manner as batch A with the exception of 5 g of No. 503 Celite was gradually added to the tissue slurry as the grinding proceeded. This thick suspension was poured on a short broad column of silica prepared by adding 2 g of Celite in suspension to a 6 cm Buchner funnel containing a No. 31 Whatman filter paper. The excess water was withdrawn with negative pressure, care being taken not to dry the Celite to the point of shrinkage. After the thick

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3. Claude, A., *J. Exp. Med.*, 1946, v84, 51.

TABLE III.
Comparison of Normal Mouse Liver Fractions Prepared by Centrifugation and by Short Column Procedure Employing 30% Sucrose.

Criteria	Extract		Resuspended pellets	
	(A) Centrifuge	(B) Short column	(A) Centrifuge	(B) Short column
Grams of liver	6	6	—	—
Vol. of extract, ml	60.0	60.0	—	—
Nitrogen, mg/ml	1.76	1.90	0.51	0.44
Succinoxidase activity				
μl O_2/hr	207	221	178	147
$QO_2(N)$	118	116	347	334
Cytochrome oxidase activity				
μl O_2/hr	272	274	187	171
$QO_2(N)$	155	144	365	388

slurry of diatomaceous silica and ground tissue was added to the prepared funnel, negative pressure was cautiously applied. Just before the column went dry an additional 25 ml of cold distilled water was added in increments to further elute the granules from the silica. The resulting percolate from lot B was diluted to 50 ml and tested against extract A. In addition to a comparison of the extracts obtained by the two methods, an aliquot of each was sedimented at high speed in the centrifuge (20,000 g for 15 minutes) so that the particulates obtained could be tested free of soluble components in the supernatant fluid. The resulting pellets were resuspended in cold distilled water to the starting volume and tested along with the extracts. By phase contrast microscopy the pellet fractions appeared to consist essentially of granules 0.2 to 1.0 μ in diameter, though some submicroscopic particulates undoubtedly were present. All fractions were tested in respect to recovery in terms of dry weight, nitrogen, phosphorus, and optical density at 650 mu; and for biological activity in terms of the dopa and succinoxidase enzyme systems. The results are given in Table I, and demonstrate that the two methods agree well in regard to dopa oxidase and succinoxidase activity and the other criteria listed.

Though this method has been employed primarily in the preparation of melanoma extracts, other tumors and mouse liver mitochondrial preparations have been explored. Preliminary results suggest that the technic may have general application with appropriate minor modifications. Tables II and III show the results obtained when employing the technic to prepare mitochondrial containing extracts from an amelanotic tumor‡ and normal mouse liver. The tumor fractions were prepared by the procedure outlined above, whereas the liver procedure was modified by employing 30% sucrose,(4) in lieu of distilled water, and by reducing the Celite used in grinding to 2.5 g. The liver extracts obtained by the two technics were microscopically similar. Both preparations contained filaments, short rods, and spherical granules, though semi-quantitative estimates with a counting chamber indicated a slightly greater population of long filaments in the centrifuge preparation. Examination of both extracts with the phase contrast microscope failed to reveal visible particulates other than those resembling mitochondrial elements. The column extract was somewhat pinker, probably due to the fact that more red blood cells were lysed by the Celite grinding. These pigments were largely eliminated, however, by subsequent centrifugation, and it has previously been shown that blood pigments can be largely, if not entirely, removed by column chromatography.(2)

Summary. The short column technic described is an adjunct to the chromatographic methods previously published(2) and makes available a complete procedure for the preparation of certain subcellular particulates from the crude tissue to the purified fractions without recourse to the centrifuge at any stage.

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‡ S91-AA tumor derived from the Cloudman S91 mouse melanoma by Algire.

4. Hogeboom, G. H., Schneider, W. C., and Pallade, G. E., *J. Biol. Chem.*, 1948, v172, 619.

Some of the advantages of the short column technic for the preliminary preparation of cytoplasmic fractions are speed, simplicity of equipment and operation, an adaptability to a controlled gas phase during manipulation, and a sharp separation of cytoplasmic elements from nuclei and viable cells.

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On the Insulin Content of Pancreatic Juice.* (17588)

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It has been suggested that the islets of Langerhans are connected with and regenerated from duct tubules.(1) It is of interest to determine whether or not the internal secretions of the islands are present in the external pancreatic secretion. We have attempted to demonstrate insulin in fresh pancreatic juice by means of the "insulin effect" on rat diaphragm *in vitro*. The uptake of glucose by the isolated rat diaphragm has been extensively studied by Gemmill,(2) Stadie and Zapp,(3) Perlmutter and Greep,(4) Villee and Hastings, (5) and others. They have shown that glycogen synthesis by the rat diaphragm (measured directly or by glucose disappearance from the medium) is increased by the addition of insulin to the substrate.

Methods. Extraction. Pancreatic juice was extracted by modification of the insulin extraction procedures of Somogyi *et al.*(6) and Best and Scott.(7) Juice from the cannulated major pancreatic duct of the dog was collected into acid alcohol (95% ethyl alcohol plus 1.3% acetic acid) in proportion two parts of juice to three parts of acid alcohol. When 400 to 600 ml had been collected (usually over two to three days) the mixture was brought to pH 7.5-8.0 with several milliliters of 1 N NaOH and filtered. The filtrate was adjusted to pH 8.0 and concentrated *in vacuo* at 25-28°C for 24 to 48 hours. The residue was taken up in 50 to 100 cc of water, brought to pH 8.0 with .01 N NaOH, and filtered. To the filtrate was added ammonium sulphate 40 g/100 ml and the precipitate allowed to settle out overnight in the ice box. The precipitate was collected in a Buechner funnel on hard filter paper and then eluted repeatedly with 80% ethyl alcohol. The addition of an equal volume of ethyl ether caused a precipitate to appear. By repeated isoelectric precipitation was isolated that fraction soluble in water at pH 3.5 and 8, and insoluble at pH 5.0. This fraction was taken up in small amounts of water, buffered, and used for assay. To certain portions of pancreatic juice commercial insulin was added and the extraction carried out as above.

Assay. Well fed adult rats were killed by hammer and diaphragms quickly excised and placed in cold saline. Quarter diaphragms weighing 50 to 100 mg were placed in Warburg flasks. Each flask contained 2.0 ml of Krebs-Ringer phosphate solution buffered to pH 7.4, and containing 100 mg% of glucose, and 0.02

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* This work has been aided by grants from the Otho S. A. Sprague Memorial Institute and from the Douglas Smith Foundation.

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TABLE I.

		Experiment	Glucose uptake mg/100 mg diaphragm/hr	% increase over control
1.	a.	Diaphragm + substrate	.111	.0
	b.	" + 0.01 μ insulin/ml	.248	124.3
	c.	" + extr. equivalent to 7.1 ml of pancreatic juice/ml substrate	.136	22.5
	d.	" + extr. of pancreatic juice to which insulin had been added .05 u./ml juice. Test extr. equivalent .9 ml juice/ml substrate	.231	108.1
2.	a.	Same as 1a	.224	.0
	b.	" " 1b	.484	116.1
		" " 1c	.284	26.8
		" " 1d	.364	62.5
3.	a.	Diaphragm + substrate	.144	.0
	b.	" + insulin 0.01 u./ml	.212	47.2
	c.	" + extr. pancreatic juice to which insulin added .05 u./ml juice. Test extr. equivalent .93 ml juice/ml substrate	.289	100.7
	d.	" + extr. 3c diluted 1/5. Test equivalent 0.18 ml juice/ml substrate	.234	62.5
	e.	" + extr. 3c diluted 1/10. Test equivalent 0.09 ml juice/ml substrate	.238	65.3
4.	a.	Diaphragm + substrate	.162	.0
	b.	" + .01 u. insulin/ml	.406	150.3
	c.	" + extr. pancreatic j. equivalent 8 ml juice/ml substrate	.200	23.1
	d.	" + extr. pancreatic j. to which insulin added .022 u./ml. Test extr. equivalent 2.2 ml j./ml substrate	.370	128.4

ml of test substance. Two-tenths ml of 10% KOH was placed in center well, flasks gassed with oxygen, and shaken 80 times per minute at 38°C for 2 hours. At termination of incubation, aliquots of media were taken for glucose determinations by modifications of Folin-Wu(8) and Shaffer-Hartmann(9) methods.

Results. In preliminary experiments, concentrations of insulin of 0.005-0.01 unit per milliliter substrate regularly increased the utilization of glucose by diaphragm from 50 to 100%. The averages for 9 animals gave glucose utilization without insulin as .150 mg/100 mg diaphragm/hour, and with added insulin of 0.01 u./ml .285 mg/100 mg diaphragm/hour. These values are in good agreement with those reported by others. We could obtain no consistent effect at concentrations of 0.001 u./ml, nor did concentrations of insulin of 0.5 u./ml increase the effect. Results of experiments with extracts of pancreatic juice are summarized in Table I.

Summary. Commercial insulin added to fresh pancreatic juice can be extracted from the juice and its effect noted on the glucose uptake by the diaphragm in quantities as small as 0.0045 unit per milliliter juice.

In extracting pancreatic juice alone, no clear-cut demonstration of insulin effect (*i.e.*, an increase in glucose uptake of 50-100%) could be obtained, even though the extracts of as much as 7 milliliters of fresh juice per milliliter of substrate were tested. (The 20 to 25% averaged increase of glucose uptake by pancreatic juice is not of statistical significance.) Thus if insulin is present in pancreatic juice, the amount extractable and detectable by our methods must be less than .005/7 or .0007 unit per milliliter of juice.

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Effect of Strychnine on the Cat's Electrocerebellogram. (17589)

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In the course of our experiments on the response of the cerebellum to various epileptogenic agents, we have observed an interesting change in the electrocerebellogram of the cat following the use of strychnine. Dow(1) has previously reported no effect on the electrical activity of the cerebellum following the application of 1% strychnine to the cerebellum except for some lowering of the amplitude and frequency of the action potentials. In one of his experiments, however, the application of 1/1000 strychnine caused the appearance of groups of large rhythmic waves at a frequency of about 70 per second. Miller(2) applied 1% strychnine nitrate to the surface of the cerebellum of cats and observed rhythmical tremors.

Methods. 75 cats were used in these studies. The animals were prepared under a brief period of ether anesthesia followed by local anesthesia with 1% procaine. The trachea was cannulated, the animal curarized, and respirations maintained artificially. Recordings were made from the cerebral cortex by removing the calvarium and applying silver-tipped electrodes to the surface of the cortex, and from the cerebellum by means of electrodes applied directly to the surface of the cerebellum, or by phonograph needle electrodes inserted through the suboccipital bone and bony tentorium. A Grass, eight-channel, ink-writing electroencephalograph was used. Following a period of control recording, 1% strychnine was injected in varying amounts intravenously, into the cerebellar cortex or into the dentate nucleus. It was also placed on the surface of the cerebellum by means of small pledgets of cottonoid.

Results. When strychnine is applied topically to the cerebellum, injected into the cerebellar cortex or dentate nucleus, or given intravenously, the electrocerebellogram develops rhythmical waves with a frequency of 10-30 per second, and a voltage of 100 to 400 microvolts occurring in periodic discharges. The time and type of onset, duration of each discharge, length of time between discharges, and persistence of the discharges depend to a great extent on the dose and mode of administration, although there is considerable variation from animal to animal (Fig. 1). The dose of strychnine required to produce an effect varied with the mode of administration. Intravenous administration or injection into the dentate nucleus of strychnine in doses of .018 to .022 mg per kilo produced in most instances rhythmical discharges. At times even smaller amounts (.011 mg per kilo) given intravenously were effective. When injected into the cerebellar cortex, approximately 0.3 mg was the necessary dose. Topical application by means of a small pledget of cottonoid soaked in 1% strychnine caused no changes, whereas the use of 2½% strychnine was effective only after 1½ to 2 hours. The time and type of onset varied. In some instances, within a few seconds after administration of the strychnine, a synchronous, rhythmical discharge appeared from the cerebellum with a frequency of 10-30 per second. The voltage increased rapidly to about 200 to 400 microvolts. In other cases changes occurred in the electrocerebellogram in from 2-30 minutes after strychnine was given. At times, the onset of a long rhythmical discharge was preceded by many minutes of intermittent bursts of 5-6 high voltage waves. This type of burst was also frequently seen between the periodic rhythmical discharges. The frequency of the discharge would also change, being perhaps 10 per second initially, gradually increasing to 15-20 per second and then gradually decreasing again. These rhythmical

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* The work described was aided by a grant from the Lederer Fund of the Johns Hopkins Hospital for study in epilepsy.

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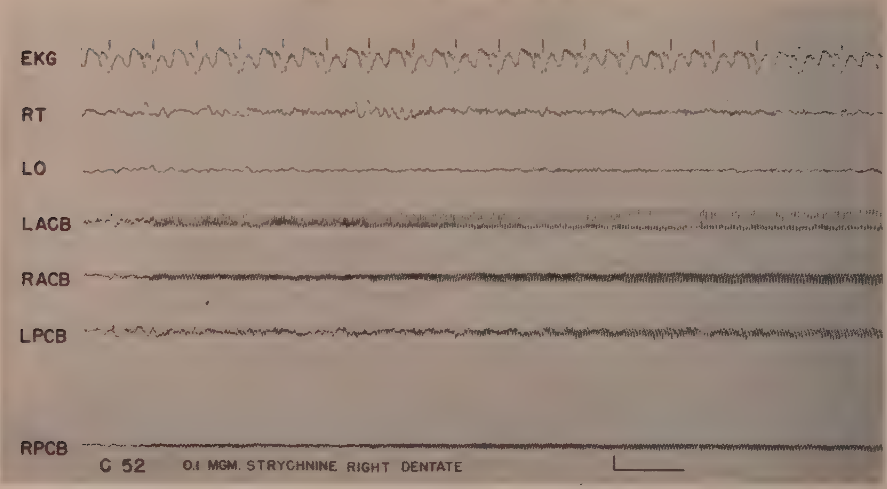

FIG. 1.

This record shows the electrocorticogram and electrocerebellogram of a cat after the injection of 0.1 mg of strychnine into the right dentate nucleus. The change in the electrocerebellogram is shown with the development of high voltage synchronous rhythmical waves. The abbreviations are: EKG, electrocardiogram; RT, right temporal region; LO, left occipital; LACB, left anterior cerebellum; RACB, right anterior cerebellum; LPCB, left posterior cerebellum; RPCB, right posterior cerebellum; LD, left dentate nucleus. The records are bipolar recordings made on a Grass electroencephalograph. The horizontal line at the base represents one second and the vertical line a calibration of 200 microvolts.

discharges lasted for varying lengths of time, from 10 seconds to as long as 2½ hours. Usually, however, the individual discharge had a duration of 30 seconds to 3 minutes, recurring at intervals of 1 to 3 minutes for 30 minutes to an hour. In one animal the rhythmical discharges recurred for a period of 4 hours.

In general, when the strychnine was given in doses just sufficient to produce a response, changes occurred in less than a minute after the intravenous injection, in a slightly longer interval after injection into the dentate nucleus, and in 10 to 15 minutes after injection into the cerebellar cortex. The changes, however, appeared to be more persistent after the intracortical injection, lasting for 1 to 2½ hours, whereas after the intravenous injection, the changes usually lasted for only about 30 minutes. Individual discharges of the highest voltage and longest duration were obtained following intravenous injection. Recordings were generally taken from the anterior and posterior surfaces of the two cerebellar hemispheres, the dentate nuclei, and from the temporal, occipital, and frontal cortex of the cerebral hemispheres. In most animals the discharge appeared from all areas of the cerebellum simultaneously. Frequently, however, the voltage was considerably higher from the dentate nucleus and the anterior surface of the cerebellum. In 2 animals the changes appeared first from these regions. These changes in the electrocerebellogram appear in the decerebrate animal as well as from the intact brain. Also, if decerebration is performed after the discharges have appeared, they continue unchanged (Fig. 2). With the amounts of strychnine used, usually an increase in frequency and a decrease in amplitude of the electrocorticogram was observed.

Discussion. Strychnine results in a different type of electroencephalographic response from the cerebellum than it does from the cerebrum. The so-called "strychnine spikes" have not been observed from the cerebellum. The site of origin of this discharge is not definitely known at this time. It appears to be an unusual type of response of the central

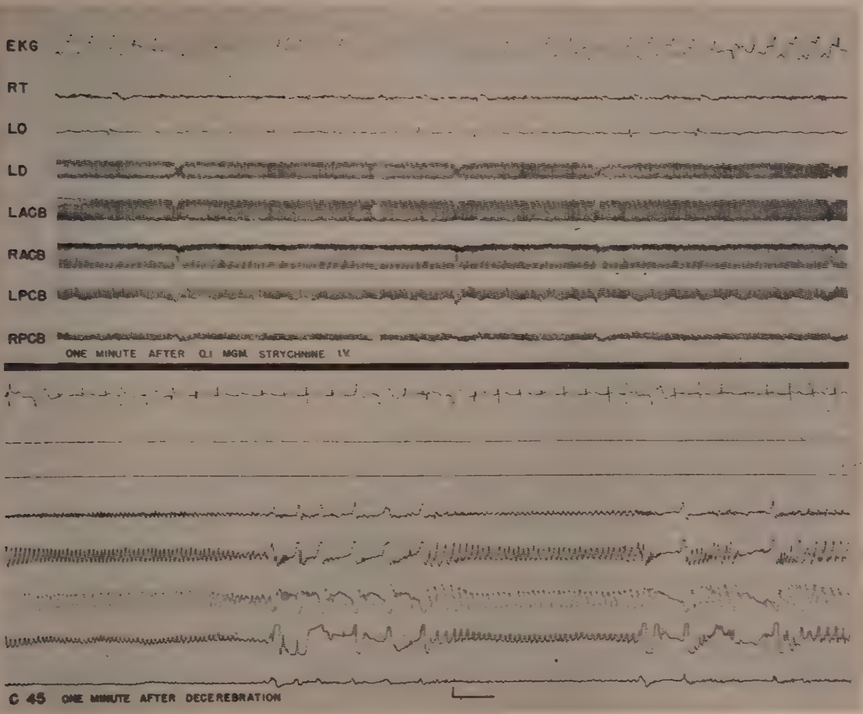

FIG. 2.

After the intravenous injection of 0.1 mg of strychnine, the electrocerebellogram of this cat shows rhythmical waves of 200 to 400 microvolts at a frequency of 24 per second. The same type of waves, although at a slower frequency, are seen to persist after decerebration. The abbreviations are the same as in Fig. 1.

nervous system to stimulation. A few preliminary experiments have indicated that the same response can be obtained from the spinal cord, medulla, and pons in the absence of the cerebellum, so that it would seem that this effect of strychnine is not peculiar to the cerebellum. It is interesting to speculate as to why the response of the cerebellum to strychnine is so different from the response of the cerebrum. The synchronous rhythmical discharge may be related to the uniform histologic structure of the cerebellum.

Summary. When strychnine is applied topically to the cat's cerebellum, injected intravenously or into the dentate nucleus in doses of .018 to .022 mg per kilo, or injected into the cerebellar cortex in doses of 0.3 mg, the electrocerebellogram develops rhythmical waves with a frequency of 10-30 per second and a voltage of 100 to 400 microvolts occurring in periodic discharges of 30 seconds to 3 minutes duration and recurring at intervals of 1 to 3 minutes for 30 minutes to an hour.

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The Nature of Rabbit Serum Cholinesterase.* (17590)

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The cholinesterases have been studied extensively in recent years especially in relation to nerve activity. However, the demonstration that more than one enzyme can split acetylcholine has disclosed the existence of a non-specific enzyme which plays no known role in nerve activity.(1) This enzyme is present in the serum and tissues of many species including man. Most of the research on it has been directed either toward the identification of this enzyme and its separation from the specific form, or toward its quantitative variation in disease states. In a previous paper,(2) we reviewed the recent literature on the variation of this enzyme in various pathological states, and concluded that in addition to our finding of a decrease of the non-specific enzyme in serum in pregnancy and patients with malignancies, there was satisfactory evidence in the literature of a decrease in the level of this enzyme, in pernicious anemia in relapse, and in liver damage. Further study in our laboratory of this phenomenon revealed that the human serum cholinesterase (the non-specific enzyme) did not vary in specific diseases, but rather varied directly with the serum albumin in all diseases except those with albuminuria. The results of this work are published elsewhere.(3)

The fact that the serum cholinesterase decreases in liver damage suggests the possibility that the liver is involved in the synthesis of both the albumin and the cholinesterase, but the exact relationship of one to the other remains to be discovered. With this in mind we went to an experimental animal, the rabbit, to work out the theoretical possibilities. The rabbit has been reported by Mendel and Rudney(4) to have both the specific and non-specific enzyme in its serum, but Ellis and co-workers(5) were unable to produce a decrease in the serum cholinesterase in this animal after liver damage. Their work was inconclusive, however, since no albumin determinations were carried out. Because of this, we decided to study the cholinesterase enzyme pattern in the rabbit before using it for our principal experiments.

Method. In the tests for serum cholinesterase, we have followed the procedure of Mazur and Bodansky,(6) who modified the method of Ammon.(7) The production of acid from the substrates was followed at 37°C by means of the liberation of carbon dioxide from a bicarbonate-carbonic acid buffer in Warburg flasks. These vessels were gassed at room temperature with a mixture of 93% oxygen and 7% carbon dioxide. At zero time, the substrate was tipped into the serum and bicarbonate mixture. Readings were made at 20 minutes at which time the readings fell on a zero order curve. 0.25 ml of rabbit serum was used in a final volume of 3.00 ml, .04 molar in sodium bicarbonate. Final substrate concentrations were: acetylcholine 0.015 M, benzoylcholine 0.006 M, and acetyl-β-methylcholine 0.03 M.

Results. The assumption that the rabbit has both the specific and non-specific cholinesterases is based on the hydrolysis of the two substrates acetyl-β-methylcholine (hydrolyzed by the specific form), and benzoylcholine (hydrolyzed by the non-specific type), in addition to acetylcholine (hydrolyzed by both). In our own experiments, the examination of the ratios of the 3 substrates revealed a fairly constant ratio of acetyl-β-methylcholine to

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* Aided by a grant from the Cancer Research Grants Division, U. S. Public Health Service.

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TABLE I.
Ratios of the Hydrolysis Rates of Benzoylcholine and Acetyl-β-methylcholine by Equal Amounts of Rabbit Serum.

Rabbit	ACh	BCh	MCh	BCh/ACh	MCh/ACh
66	28.7	9.5	13.3	.33	.47
69	39.1	34.3	16.5	.96	.42
70	52.8	32.7	21.7	.62	.41
52	39.4	6.7	15.4	.17	.39
59	35.7	20.9	11.2	.59	.32
60	34.7	18.6	11.7	.54	.34
64	32.4	4.8	11.1	.15	.35
122	48.6	71.3	15.7	1.5	.32
Aero.	62.0	39.4	27.6	.64	.45
57	62.7	81.4	19.0	1.3	.30
55	36.5	30.1	12.5	.82	.34
54	43.1	55.2	18.2	1.3	.42
53	39.4	29.3	19.3	.74	.49
Gaud.	43.6	47.9	19.8	1.1	.46
81	53.5	27.1	19.2	.51	.36
83	39.4	33.2	11.7	.84	.30
84	48.6	39.1	18.8	.81	.39
85	62.8	30.6	24.4	.49	.39
87	49.0	33.2	14.8	.67	.30
88	52.6	44.5	21.5	.85	.41
89	56.9	43.2	23.6	.76	.42
90	66.5	41.1	23.1	.62	.35
91	62.0	112.0	23.8	1.8	.30
82	48.2	42.9	21.0	.89	.44
93	47.2	5.2	22.8	.11	.49
94	48.7	35.0	17.6	.72	.37

TABLE II.
Effect of Pontocaine upon Cholinesterase Activity.*

Source of enzyme	Inhibition of hydrolysis of		
	Acetylcholine %	Benzoylcholine %	Acetyl-B-methylcholine %
Human Serum	100	2	—
Hemolyzed Human Erythrocytes	0	—	0
Rabbit Serum	2	7	11
Hemolyzed Rabbit Erythrocytes	4	—	12

* Final concentration of pontocaine hydrochloride 10^{-4} M.

acetylcholine hydrolysis, regardless of whether the rabbit serum contained an enzyme hydrolyzing benzoylcholine or not. On the other hand, there was no constant relationship between the benzoylcholine and acetylcholine hydrolysis (Table I). This would indicate that the enzyme which hydrolyzes benzoylcholine in the rabbit either does not hydrolyze acetylcholine or at best hydrolyzes it in insignificant quantities. This is unlike the human non-specific serum cholinesterase, which gives a constant ratio of the hydrolysis of benzoylcholine to acetylcholine. The average ratio of benzoylcholine to acetylcholine hydrolysis, for equal amounts of serum, for 42 normal individuals, as determined in this laboratory, was 0.345 with a standard deviation of 0.04. Thus we must conclude that the enzymes hydrolyzing benzoylcholine in the rabbit and the human are different and that measurement of acetylcholine hydrolysis alone would yield no information in the rabbit as to the enzyme hydrolyzing benzoylcholine.

Further evidence that the enzymes hydrolyzing acetylcholine in the human and in the rabbit are different, and that very likely only the enzyme hydrolyzing acetyl-β-methylcholin attacks acetylcholine in the rabbit is presented in Table II.

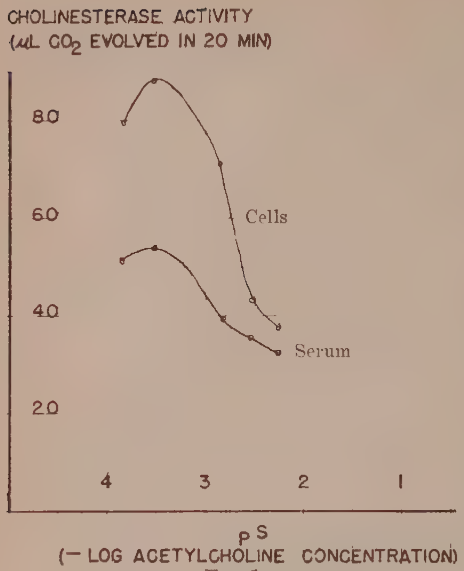

FIG. 1.
Activity-pS curves for rabbit serum and erythrocytes.

Ferrarai(8) has shown that pontocaine inactivates the non-specific cholinesterase but not the specific cholinesterase. Since the results in Table II indicate no inhibition by pontocaine of acetylcholine hydrolysis by rabbit serum, we must conclude that the enzyme hydrolyzing acetylcholine in the rabbit is not the same one hydrolyzing it in human serum.

Evidence that the serum enzyme in the rabbit is the true type of cholinesterase is seen in Fig. 1. The activity-pS curve here is the bell-shaped curve characteristic for the specific or true cholinesterase and acetylcholine.(9) This may further be seen from the curve for the rabbit erythrocytes also presented, the erythrocytes of most mammalian species having the true cholinesterase.

Conclusions. Rabbit serum, contrary to previous observations, contains only one enzyme which hydrolyzes acetylcholine, and this one has the characteristics of a true cholinesterase. In addition, there is present in rabbit serum an enzyme which hydrolyzes only benzoylcholine, and which, since it does not hydrolyze acetylcholine significantly, should not be labeled a cholinesterase. This enzyme may be similar to the "benzoylcholinesterase" described by Sawyer(10) as being present in guinea pig liver and kidney.

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Renal Hyperemia Induced in Man by a New Phthalazine Derivative.
(17591)

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Pyrogen is the only substance which has been reported to increase significantly the renal blood flow in man. However, according to Goldring and Chasis,(1) it is not suitable for therapeutic purposes. Aminophylline seems to be effective only in patients with cardiac failure.(2) No treatment which will relieve renal ischemia has yet been found.

According to Meier *et al.*,(3) Compound 5968 (hydrazino-phthalazine hydrochloride), supplied by Ciba, produces in animals a sustained fall in blood pressure. In man, we found that hypotension did not occur regularly following the subcutaneous injection of 10 mg. However, when measurements of renal plasma flow were performed, using the

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TABLE I.
Changes in Renal Hemodynamics of Human Subjects Induced by Subcutaneous Injections of 5968 (Ciba).

Diagnosis	Dose (mg)	Before*				After†				Change in C_{PAH}‡
		C_{PAH}	C_T	F.F.	B.P.	C_{PAH}	C_T	F.F.	B.P.	
Ess. hypertension	20	429	128	.30	200/115	593	116	.20	175/70	+38
,, ,,	12	432	86	.20	180/100	628	108	.17	180/100	+45
,, ,,	10	405	71	.17	170/80	555	74	.13	160/55	+36
,, ,,	10	315	84	.27	160/90	530	104	.20	160/80	+68
,, ,,	10	352	118	.34	160/80	469	126	.28	135/65	+33
,, ,,	8	606	98	.16	140/95	850	79	.09	130/90	+40
Chronic nephritis	10	182	32	.18	175/85	233	35	.15	130/70	+28
,, ,,	8	822	125	.15	145/75	950	114	.12	145/17	+16
Normal	9	730	140	.19	125/75	911	169	.19	115/75	+25
,,	9	628	104	.17	110/60	884	129	.15	110/50	+41
,,	9	733	149	.20	130/95	1167	143	.12	130/80	+59
,,	8	720	153	.21	125/80	892	173	.19	125/65	+24

* Each value represents average of 3 control periods.
† Maximal changes observed after injection.
C_{PAH}: Sodium para-aminohippurate plasma clearance (cc/min.).
C_T: Sodium thiosulphate plasma clearance (cc/min.).
F.F: Filtration fraction.
B.P.: Blood pressure (mm of mercury).
Ess. Hypertension: Essential hypertension.
‡ Expressed as per cent of control value.

clearance technic,(1) it became evident that renal hyperemia could be elicited in nearly every subject. A similar effect has also been demonstrated in cats by thermostromuhr measurements.(3)

Our data, presented in Table I, demonstrate that this drug can cause increase in renal plasma flow in man varying from 16 to 68%, averaging 38.6%. The glomerular filtration rate, as reflected by the clearance of thiosulphate, is not consistently affected. The filtration fraction is always decreased. In some cases, urine volumes are markedly increased. Blood pressure shows a definite reduction only in some hypertensive patients. Therefore, the influence of the drug upon the kidney is somewhat similar to that of pyrogenic substances. The responses obtained are moderately prolonged, lasting 1 to 2 hours or more. Toxic manifestations are relatively unimportant. Moderate tachycardia may be observed in some cases. Effects of the drug upon cardiac output are being investigated.

From these results, it can be concluded that the drug 5968 (Ciba) is a renal vasodilator in man.

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Effect of Thiazine, Oxazine and Phenazine Dyes on Normal and Heparinized Rabbit Plasma.* (17592)

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The recent work of Allen and Jacobson(1) showing the usefulness of Toluidine Blue as a heparin inactivator in x-irradiation damage has revived interest in the field of synthetic heparin inactivators. Haley and Stolarsky(2) using MacIntosh's(3) *in vitro* method evaluated 19 dyes and found that only those belonging to the thiazine, oxazine and phenazine series which had a free primary amine group were active heparin inactivators. This type of chemical evaluation, while it determines quantitatively the amount of heparin precipitated and removed from solution, does not give any indication of biological heparin inactivation. Such chemical studies must be supplemented by investigations of the effect of heparin inactivators upon whole blood or plasma *in vitro*. This type of investigation is extremely important because it has been reported that small amounts of Toluidine Blue increase the anticoagulant effect of heparin while larger amounts of the dye decrease coagulation time but do not entirely inactivate heparin.(4,5) Similar results have been reported for Safranine, Neutral Red and Janus Green.(6-8) However, these results do not cover the entire group of effective heparin inactivating dyes nor do they give exact quantitative data. We decided to investigate the effect of our most potent chemical inactivators of heparin(2) upon the *in vitro* coagulation of plasma in an attempt to quantitate the concentration of dye required to return the coagulation time to the normal control level and also determine the overall effect of these dyes upon the coagulation mechanism.

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* This paper is based on work performed under Contract No. AT-04-1-GEN-12 between the Atomic Energy Commission and the University of California at Los Angeles.

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Experimental. Blood was obtained from 6 rabbits by cardiac puncture every 2 weeks. The pooled blood was mixed with 3.8 per cent citrate at a ratio of 1:4, centrifuged and the supernatant plasma removed and stored at 4°C until used. During the preliminary phase of the work any plasma showing fibrin threads was discarded. In the latter phases we found, in confirmation of Lewis and De Maria,(9) that filtration through glass wool removed any fibrin present and gave a plasma with good coagulation properties. A modified Astrup(10) method was used for studying the effect of the various dyes on the coagulation time of normal and heparinized rabbit plasma. The following solutions were employed: Difco Thromboplastin 0.01% in normal saline, calcium chloride ($CaCl_2 \cdot 2H_2O$) 0.68% in distilled water, and powdered heparin sodium Upjohn (120 Toronto Units/mg) 0.1% in normal saline. The majority of the dyes were made up in concentrations of 5 to 200 μg per 0.1 cc. Janus Green gave a precipitate above 25 μg per 0.1 cc making it impossible to study the effect of higher concentrations of this dye. Neutral Red was used up to its maximum solubility, 800 μg per 0.1 cc. Thionine was not soluble above 25 μg per 0.1 cc and Safranine above 100 μg per 0.1 cc. The usual solubilizing procedures, ethanol and acid, resulted in inactivation of the clotting mechanism. The test tubes (10 x 100 mm) used were left in cleaning solution for 24 hours, rinsed 3 times with tap water followed by three rinsings with distilled water, then dried and stored until used. Coagulation studies were performed as given in Table I, the solutions mixed in the order of their column. Prior to mixing, the separate solutions were kept in an ice bath at about 1°C and after mixing in a water bath at 37.5°C for 60 seconds. All determinations were performed in triplicate and the end point used was the first appearance of fibrin threads. These threads are easily seen when direct light passes through the solution.

Results. The protocol of a typical experiment showing the effect of Toluidine Blue upon normal and heparinized rabbit plasma is given in Tables II and III. Graph 1 illustrates the effect of the seven dyes investigated upon the clotting times of normal and heparinized rabbit plasma. In all cases the dyes tended to produce a rapid inactivation of heparin although the amount of dye required to return the clotting time to its normal level varied with the particular dye used. It is apparent that the amount of dye required for heparin inactivation is critical because small

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TABLE I.
Order of Mixing of Coagulation Systems.

Tube	Plasma, cc	Thrombo-plastin, cc	Distilled water, cc	Heparin, cc	Dye, cc	CaCl, cc
Control	.1	.05	.1	0	0	.2
Heparin	.1	.05	0	.1	0	.2
Dye	.1	.05	0	0	.1	.2

TABLE II.
Coagulation Time in Seconds of Plasma and Toluidine Blue.

Concentration of dye, μg/0.1 cc (0.85% NaCl)	0	5	10	15	25	50	75	100	125	150	200
1st series of tubes	92	89	92	92	104	135	191	215	298	330	376
2nd series of tubes	92	86	90	93	105	136	186	213	293	319	376
3rd series of tubes	93	87	94	97	107	138	195	209	289	327	382
Avg time in sec.	92	87	92	94	105	136	191	212	293	325	378

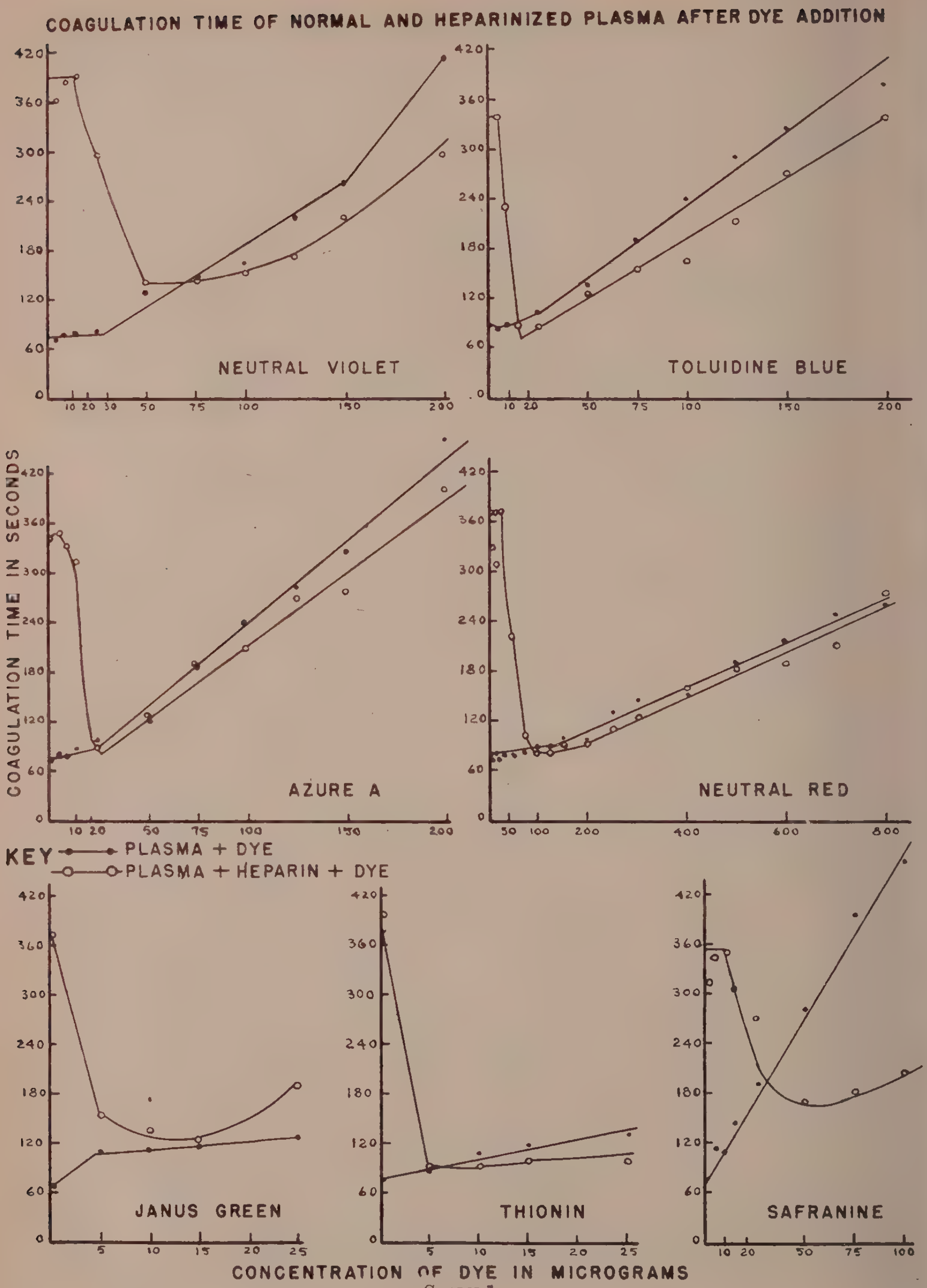
TABLE III.
Coagulation Time in Seconds of Plasma, Heparin, and Toluidine Blue.

Concentration of dye, μg/0.1 cc (0.85% NaCl)	0	5	10	15	25	50	75	100	125	150	200
1st series of tubes	350	353	293	89	96	136	170	172	205	270	293
2nd series of tubes	354	356	296	88	100	140	165	169	217	268	291
3rd series of tubes	355	350	290	91	98	142	164	173	217	272	308
Avg time in sec.	353	353	293	89	98	139	166	171	212	270	297

amounts do not greatly reduce clotting time, whereas larger quantities prolong clotting time. Inspection of the curves in Graph 1 shows that after dye addition beyond the critical point, except in the cases of Janus Green, Thionine and Safranine, the curves for both the normal and heparinized plasmas are essentially parallel. This would indicate that these dyes have an anticoagulant effect and that the excess dye is acting the same in both cases and upon some substance in the system other than heparin.

Discussion. It has been reported previously that when small amounts of Toluidine Blue (4,5) are added to heparinized blood the coagulation time is prolonged. We were unable to confirm these findings; in fact, we found that this dye had no effect on normal plasma at concentrations of 5 and 10 μg. However, this latter concentration markedly reduced the coagulation time of heparinized plasma. The anticoagulant effect observed with increasing concentrations of Toluidine Blue(4,5) and Janus Green(6-8) is essentially the same as reported herein. Herrmann(6) reported that Neutral Red did not increase coagulation time but we found that this dye behaved as an anticoagulant when the concentration was sufficiently high. The results herein reported for Neutral Red and Safranine confirm those of von Jeney, *et al.*(8) However, those investigators worked with only one concentration of dye, 100 μg per 0.1 cc, and therefore did not observe the anticoagulant effects we are reporting. It appears from the results herein presented that the indiscriminate use of these dyes in heparinemia would be highly detrimental to the welfare of a patient. However, the *in vivo* effects of these dyes may not be entirely the same as the *in vitro* effects.

Summary. A study has been made of the effect of 7 dyes of the phenazine, oxazine and thiazine series on the *in vitro* coagulation times of normal and heparinized rabbit plasma. Toluidine Blue, Azure A, Neutral Violet and Neutral Red effectively counteract the anticoagulant action of heparin but the amount of each dye required for this antiheparin effect is


COAGULATION TIME OF NORMAL AND HEPARINIZED PLASMA AFTER DYE ADDITION
COAGULATION TIME IN SECONDS
0
60
120
180
240
300
360
420
NEUTRAL VIOLET
10 20 30 50 75 100 150 200
TOLUIDINE BLUE
10 20 50 75 100 150 200
AZURE A
10 20 50 75 100 150 200
NEUTRAL RED
50 100 200 400 600 800
KEY
PLASMA + DYE
PLASMA + HEPARIN + DYE
JANUS GREEN
5 10 15 20 25
THIONIN
5 10 15 20 25
SAFRANINE
10 20 50 75 100
CONCENTRATION OF DYE IN MICROGRAMS

Graph 1.

critical. At concentrations below the critical level the dyes had little or no effect upon the coagulation system studied. At concentrations above the critical level all the dyes produced a progressively increased incoagulability of the system. This anticoagulant effect of the dyes is related to concentration and the action is on some substance other than heparin.

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Toxicity of Some Synthetic Pteridines in Rats.* (17593)

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This is an extension, into the field of vitamin analogs, of the research started in this laboratory(1) on the effect of amino acid analogs on tumor-bearing rats and mice. While there is considerable literature on the anti-folic acid activity and tumor-inhibiting effects of such folic acid analogs as 4-amino-pteroylglutamic acid and 4-amino-N-10-methyl pteroylglutamic acid, less work has been done with the substituted pteridines themselves. The importance of the 4-amino substitution for folic-acid antagonism has been demonstrated in much of this work.(2) In this connection, the anti-folic acid activity and tumor-inhibiting effect of 2,6-diamino-purine is of interest.(3) This compound is identical with 2, 4-diamino-pteridine except for the extra carbon atom in the pteridine ring. In view of these facts, several 4-amino substituted pteridines were prepared in this laboratory.(4) A study of the toxicity of these compounds in normal rats was undertaken before investigating their effects on tumor-bearing animals.

Procedure. For this study, 56 female rats of the Long-Evans strain weighing about 150 g were used. They were divided into 7 groups of 8 animals each. Two groups served as controls, while each other group received one of the 5 different pteridines. They were kept on the regular stock diet during the experimental period. The compounds were dissolved in 0.1 M phosphate buffer, pH 7.4, and the concentrations used, except for 2,4-diamino-pteridine, approached the maximum solubility.

Of the eight animals used for each compound studied, 4 received 3 ml intraperitoneally daily (Low Dose), and 4 received 3 ml twice daily (High Dose). One control group was not injected; the other was given the same volume of phosphate buffer as the experimental animals. The injection period was for 10 days. Half of each group was killed on the eleventh day (Group A), and the rest were killed 6 days later (Group B). At the time of sacrifice, blood was taken for hemoglobin, white blood count and blood smear. The kidneys and liver were weighed, and pieces of kidney, liver and sternal marrow were fixed in formalin. A bone marrow smear was made by

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† U. S. Public Health Special Fellow.

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TABLE I.
Total Doses of Pteridines Injected.

Compound	Concentration (mg/100 ml)	Total dose in 10 days Low (mg)	Total dose in 10 days High (mg)
2-thiol-4-amino-pteridine	4	1.2	2.3
8-thiol-4-amino-6,7-dimethyl-pteridine	5	1.5	2.9
2-thiol-4-amino-6,7-dihydroxy-pteridine	100	30	57
2,4-diamino-pteridine	100	30	57
2,4-diamino-6,7-dihydroxy-pteridine	4	1.2	2.3

TABLE II.
Effects of Injection of Pteridines on Animal Weight.

Compound injected	Group and No. of animals, per group	Initial wt, g*	Gain during 10-day inj., g*	Gain in following 6 days, g*
Controls, no inj.	A + B (8)	154 ± 2.6	21.3 ± 3.6	
	B (4)	154 ± 5.4	26.0 ± 3.6	7.0 ± 2.0
Controls, buffer	A + B (8)	144 ± 2.4	22.8 ± 2.8	
	B (4)	142 ± 4.2	24.5 ± 3.5	7.0 ± 3.0
2-thiol-4-amino-pteridine	A + B (8)	156 ± 1.6	18.9 ± 2.2	
	B (4)	158 ± 2.7	22.8 ± 2.6	3.8 ± 2.7
2-thiol-4-amino 6,7-dimethyl-pteridine	A + B (8)	146 ± 2.3	20.9 ± 4.5	
	B (4)	145 ± 2.1	20.3 ± 4.6	9.0 ± 2.0
2-thiol-4-amino-6,7-dihydroxy-pteridine	A + B (8)	158 ± 4.7	19.6 ± 2.3	
	B (4)	160 ± 4.4	20.5 ± 4.8	7.8 ± 2.2
2,4-diamino-pteridine	A + B (8)	152 ± 2.3	7.9 ± 1.8	
	B (4)	150 ± 3.0	11.0 ± 2.7	8.0 ± 1.7
2,4-diamino-6,7 dihydroxy-pteridine	A + B (8)	143 ± 2.7	21.3 ± 2.4	
	B (4)	138 ± 3.5	21.8 ± 4.3	—11.5 ± 1.8

* Values represent arithmetic mean, ± the standard error of the mean.

forcing a drop of plasma through a segment of rib. In some animals the kidney tissue was also fixed in Carnoy's solution (chloroform, alcohol, and glacial acetic acid).

Results. Effects on animal body weight. The animals were weighed every other day. Table II shows the average initial weight, the average gain during the injection period, and the average change in weight during the following 6 days.

The significant findings were, 1) the 2,4-diamino-pteridine group gained less weight during the injection period but continued to gain at the normal rate during the following 6 days, and 2) the 2,4-diamino-6, 7-dihydroxy-pteridine group lost weight during the 6 days following the injections.

Effects on kidney and liver weight. No compound produced a consistent increase or decrease in the weight of either organ. However, because of the number of animals used, such effects would not be detected unless they were striking.

Hematological effects. Hemoglobin was determined by the acid hematin method, using a Klett photoelectric colorimeter. No tendency toward anemia was observed in any group. For the differential count 200 cells were examined. No significant effects were seen in the white blood counts nor the differentials. However, two of the 2,4-diamino-pteridine animals showed a depressed total white blood count and a relative granulocytopenia. Because of this, another group of 6 similar animals, with 6 controls, was given the high dose of 2,4-diamino-pteridine under the same experimental conditions, except that blood counts were done on every third day during the injection period. No effects on the white counts nor the differentials were observed in this group.

Histological effects. No pathological changes were found in any of the liver sections nor in the bone marrow smears and sections. The important changes were confined to the kidneys of those animals receiving 2,4-diamino-pteridine. All showed deposits of yellow, burr-like crystals within the renal tubules. The deposits were detectable grossly, on the cut surface of the kidneys containing the greatest amounts of crystals, as a yellow discoloration most marked in the outer zone of the medullary pyramid or at the cortico-medullary junction. This area fluoresced strongly under ultra-violet light. Microscopically, none of the spherical, radially striated crystals were found in the glomeruli. They appeared in increasing numbers in the lower parts of the nephron, and were most numerous in the collecting tubules. Yellow intracellular deposits, not definitely crystalline, were found in an occasional tubule cell. In some tubules the lumen was completely blocked by an accumulation of the crystals, and in such areas the epithelial cells were flattened, the nuclei pyknotic, and inflammatory cells had infiltrated the region. No evidence of increased mitosis was found.

The amount of crystalline deposits and the extent of the renal lesions were greater in those animals receiving the higher dose and in those killed immediately after the injection period. The deposits were better preserved with Carnoy's fixation than with formalin since the latter dissolved away some of the crystals.

Discussion. The weight loss in the animals receiving 2,4-diamino-pteridine was correlated with the degree of crystalline deposits in the kidneys. This was observed even more clearly in a previous experiment using a wider range of dosage. It appears probable that the observed effect of this compound on body weight can be ascribed either entirely or principally to the rênal damage. Crystal deposits within the renal tubules have been reported following administration of xanthopterin (2-amino-4,6-dihydroxy-pteridine)(5) and high doses of folic acid itself.(6)

Since this was a study of general toxicity rather than an investigation of antifolic acid action of the compounds, no attempt was made to lower the level of folic acid in the diet nor to reduce the intestinal bacterial flora. Swenseid, *et al.*(7) reported leukopenia but no anemia in succinyl-sulfathiazole-treated weanling rats receiving 2,4-diamino-6,7-diphenyl-pteridine at a level of 50 mg per 100 g in the diet. However, the dimethyl-substituted compound, 2,4-diamino-6, 7-dimethyl-pteridine, was much less toxic. It is not unreasonable to expect that 2,4-diamino-pteridine itself would have even less effect on the hematopoietic system. Daniel, *et al.*(8) found no anemia nor growth impairment in chicks fed 2,4-diamino-pteridine at a level of 200 mg per 100 g in the diet, along with approximately 15 γ of folic acid per 100 g of diet.

Summary. Five 4-amino-pteridine compounds were studied for toxicity in rats. The 2-thiol-substituted pteridines were not found to be toxic in the doses used. The 2,4-diamino-6,7-dihydroxy-pteridine and 2,4-diamino-pteridine interfered with the growth rate. The latter compound caused crystalline deposits in the renal tubules, and the extent of the deposits correlated well with the body weight effect. No histological effects were noted in the liver and bone marrow,‡ and none of the compounds altered the blood picture.

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‡ We wish to express our appreciation to Mr. Robert Goldberg for advice and assistance in the histological work.

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Microbiological Assay with *Lactobacillus arabinosus* after Storage for Three Years Lyophilized and in Conservation Medium.* (17594)

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Nymon *et al.*(1) have reported that the response of *Lactobacillus casei* and *Lactobacillus arabinosus* to riboflavin and niacin is essentially unchanged by the lyophile process and by storage for 3 months in the dehydrated condition. Other workers (Riesen *et al.*)(2) have shown that lyophilized cultures of lactic acid bacteria remain viable during a year of storage. Although it has been reported in the *Difco Manual*(3) that lactobacilli may be kept for 6 months in a cooked meat medium, it does not appear that maintenance of lactic acid bacteria in this or other conservation media has been employed with stock cultures for microbiological assays. Since it may be advantageous in some laboratories to maintain assay organisms by one of these methods rather than by frequent subculture, it seemed desirable to determine whether such maintenance might influence the performance of bacteria in microbiological assays. In the present study cultures of *Lactobacillus arabinosus* stored approximately 3 years lyophilized and in conservation medium have been employed to determine leucine in casein, gelatin, silk fibroin, and an amino acid mixture.

Experimental. The stock culture of *Lactobacillus arabinosus* was carried as described by Dunn *et al.*(4) One transplant from the stock culture was lyophilized essentially according to the procedure of Nymon *et al.*(1) and stored at room temperature (averages, 23°C) for 32 months. A second transplant was incubated 24 hours at 35° in Bacto-cooked meat medium. The culture tube was tightly closed with a screw cap and allowed to stand at room temperature for 38 months. After the stipulated storage periods the two cultures were transferred by stab to Bacto-tomato juice agar (Difco product),† and were stored in the refrigerator (4°) a few days before use. In preliminary experiments inocula were prepared by transferring directly from the stab cultures to the inoculum medium (Dunn *et al.*)(4) In the other experiments all 3 cultures were carried through 10 consecutive daily subcultures in the inoculum medium before use. For convenience the stock culture, lyophilized culture and cooked meat medium culture have been designated as cultures S, L, and CM, respectively. The assay methods for leucine and valine were those of Dunn *et al.*(4) The results are given in Fig. 1 and 2 and in Tables I and II.

Discussion. Leucine and valine standard curves obtained with the 3 cultures in a preliminary experiment are shown in Fig. 1 and 2. It may be seen that the curves were nearly identical with cultures S and L, but were considerably lower with culture CM, indicating that storage in the cooked meat medium may have reduced the vitality of this culture. Since the work of Nymon and Gortner(5) suggested that such a condition might be alle-

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* Paper 63. For Paper 62, see Dunn and McClure.(7) This work aided by grants from the American Cancer Society through the Committee on Growth, National Research Council. The authors are indebted to Samuel Eiduson and Gene Molene for technical assistance. Thanks are due to Harriette Block and Dr. S. Shankman for preparation of the cultures for storage.

† The lyophilized culture was rehydrated with the addition of 2 ml of inoculum medium (Dunn *et al.*(4) and incubated 24 hours at 35° before transferring to the agar.

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TABLE I.
Response of Three Cultures of *L. arabinosus* to Leucine in Casein Hydrolysate.*

Hydrolysed casein,† γ/tube	Ml 0.09 N NaOH to titrate contents of triplicate tubes‡			% leucine found†		
	Culture CM	Culture S	Culture L	Culture CM	Culture S	Culture L
74.6	4.00	5.29	5.39	9.54	9.22	9.33
149.2	6.33	8.45	8.57	9.15	9.17	9.06
223.8	8.41	11.02	11.19	9.31	9.06	9.35
298.4	10.19	13.27	13.27	9.31	9.31	9.52
373.0	11.80	14.62	14.57	9.25	9.62	9.57
			Avg	9.31	9.28	9.37

* Culture CM was stored 38 months at room temperature in cooked meat medium, culture S was the stock culture control, and culture L was stored 32 months at room temperature in the lyophilized condition.

† Values calculated for the moisture and ash-free product.

‡ Titration procedure described by Dunn *et al.*(4)

TABLE II.
Leucine Found in Proteins and Amino Acid Mixture by Microbiological Assay with Three Cultures of *L. arabinosus.**

Sample	Leucine found or recovered†					
	Culture CM		Culture S		Culture L	
	%	MDM‡	%	MDM‡	%	MDM‡
Casein	9.3	.09	9.3	.15	9.4	.14
Silk fibroin	.79	.007	.81	.013	.83	.019
Gelatin	3.1	.02	3.2	.02	3.2	.06
Amino acid mixture§	97	.7	101	1.5	104	1.6

* Culture CM was stored 38 months at room temperature in cooked meat medium, culture S was the stock culture control, and culture L was stored 32 months at room temperature in the lyophilized condition.

† Values calculated for protein on moisture and ash-free basis.

‡ Mean deviation from the mean of values obtained at 5 dosage levels.

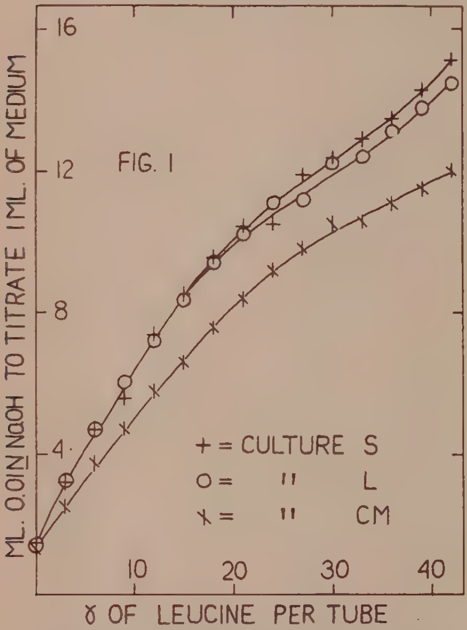
§ 4.85% L-leucine. DL-Alanine, L-aspartic acid, L-arginine · HCl, L-cystine, L-glutamic acid, glycine, L-histidine · HCl · H_2O, DL-isoleucine, L-lysine · HCl, DL-methionine, DL-phenylalanine, L-proline, DL-serine, DL-threonine, L-tyrosine, and DL-valine were present in proportions similar to those found in animal proteins (Dunn *et al.*) (4)

viated by a series of frequent subcultures in a nutrient-rich medium, all three cultures were carried through 10 consecutive daily transfers in the inoculum medium of Dunn *et al.*(4) No change was noted in any of the cultures, however, and the final turbidity attained in the liquid medium by culture CM was only about two-thirds that produced by cultures S and L throughout the series of transfers.

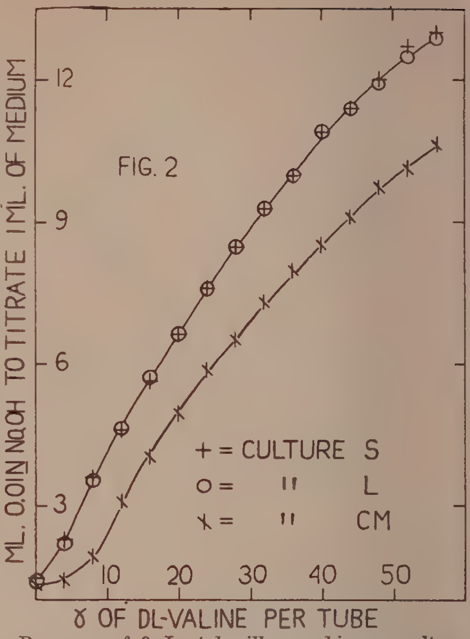
Nymon *et al.*(1,5) have implied that the quality of microbiological assays may be predicted from the appearance of the standard assay curves. According to this view it might be anticipated that microbiological assays with culture CM would be of relatively poor quality. The assay values given in Tables I and II failed to support this hypothesis, however. The agreement of results with the 3 cultures was well within anticipated experimental error, although acid production was considerably lower with culture CM (Tables I and II). The percentages found or recovered in assays with cultures CM, S, and L, respectively, were as follows: casein, 9.3, 9.3, 9.4; silk fibroin, 0.79, 0.81, 0.83; gelatin, 3.1, 3.2, 3.2; and amino acid mixture,‡ 97, 101, 104. It may be noted that these values are in close agreement with each other and with the values obtained previously for the same protein samples with *Lactobacillus arabinosus* and a different assay medium (Camien and

Dunn).(6)

It appears, therefore, that the slope of the assay curve and the quantity of acid produced by the test microorganism may be relatively unimportant provided that nearly optimal assay conditions and medium are employed. This is contrary to the view of Nymon and Gortner(5). It may be concluded that *Lactobacillus arabinosus* and perhaps other lactic acid bacteria can be stored successfully for relatively long periods lyophilized or in cooked meat medium. The lyophile process may be preferable to the conservation medium method since the former procedure apparently does not affect the acid producing ability of the organism.



Response of 3 *Lactobacillus arabinosus* cultures to L-leucine. Culture CM was stored 38 months at room temperature in cooked meat medium, culture S was the stock culture control, and culture L was stored 32 months at room temperature in the lyophilized condition. The assay method was that of Dunn *et al.*(4)



Response of 3 *Lactobacillus arabinosus* cultures to DL-valine. Culture CM was stored 38 months at room temperature in cooked meat medium, culture S was the stock culture control, and culture L was stored 32 months at room temperature in the lyophilized condition. The assay method was that of Dunn *et al.*(4)

Summary. Cultures of *Lactobacillus arabinosus* have been stored at room temperature for 32 months lyophilized and for 38 months in Bacto-cooked meat medium (Difco product). The assay performance of the cultures was not impaired by this treatment, although acid production in the cooked meat medium culture was reduced. It was concluded that these methods of storage may be substituted successfully for frequent subculture with *Lactobacillus arabinosus* and perhaps other lactic acid bacteria.

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‡ 4.85% L-leucine. DL-alanine, L-aspartic acid, L-arginine · HCl, L-cystine, L-glutamic acid, glycine, L-histidine · HCl · H_2O, DL-isoleucine, L-lysine · HCl, DL-methionine, DL-phenylalanine, L-proline, DL-serine, DL-threonine, L-tyrosine, and DL-valine were present in proportions similar to those employed previously (Dunn *et al.*) (4)

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A Method for Producing Chronic Cardiac Failure in Dogs.* (17595)

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The difficulty of producing slowly progressive, chronic, congestive failure in lower animals has retarded the analysis of the factors involved in the pathogenesis of cardiac failure. Acute decompensation has been produced by a number of methods (see Roos and Smith(1), but attempts to simulate the chronic form have met with limited success.(2-4) Many workers have found that if the heart were sufficiently damaged to produce overt decompensation, the animals would die acutely, whereas lesser grades of damage were not followed by failure at rest. However, Landis *et al.*(5) have shown in acute experiments that minor decreases in cardiac competence, which are not apparent at rest, may lead to elevations of central venous pressure during exercise, with its increased venous return. They have suggested that these transient elevations of venous pressure during muscular activity may play an important role in the development of the signs and symptoms of congestive failure.

In order to investigate this relation between cardiac competence, exercise and venous pressure in the unanesthetized animal we have studied the changes of auricular pressure of dogs during exercise on the treadmill before and after the production of experimental cardiac damage. In the initial approach to this problem the operative lesions were limited to the right side of the heart. The following lesions were produced: (a) insufficiency of the pulmonary valve, (b) stenosis of the pulmonary artery,(6) (c) the combination of pulmonary stenosis and pulmonary insufficiency, (d) insufficiency of the tricuspid valve, and (e) the combination of tricuspid insufficiency and pulmonary stenosis. During the course of this study, which will be reported fully at a later date, it was found that chronic, congestive failure resulted from the combination of tricuspid insufficiency and pulmonary stenosis. Three dogs so treated developed cardiac failure, whereas the other lesions did not lead to overt decompensation, even with strenuous exercise. This paper describes the method for producing a form of chronic, "right-sided" cardiac failure. The salient data from the experiments on those dogs that developed congestive failure are also briefly presented.

Methods. Each dog was trained and conditioned on the treadmill with repeated observations of his work tolerance. Then, a standard control experiment was performed for comparison with the postoperative response under the same work conditions. A radiopaque catheter was inserted into the auricle through a cannula in the external jugular vein. The tip of the catheter, located fluoroscopically, was used as the zero reference point. Mean right auricular pressure was measured with a water manometer. Heart rate was recorded by a cardiotachometer. The dogs were exercised on the treadmill for successive 10-minute periods at increasing speed and grade (bottom Fig. 1) until exhausted.

Following one or more control experiments, insufficiency of the tricuspid valve was produced in each dog under nembutal anesthesia, and during positive pressure respiration. In 2 of the dogs the left thorax was entered through the fourth interspace, the heart rotated slightly to the left, purse-string sutures placed in the

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† Fellow of the National Research Council.

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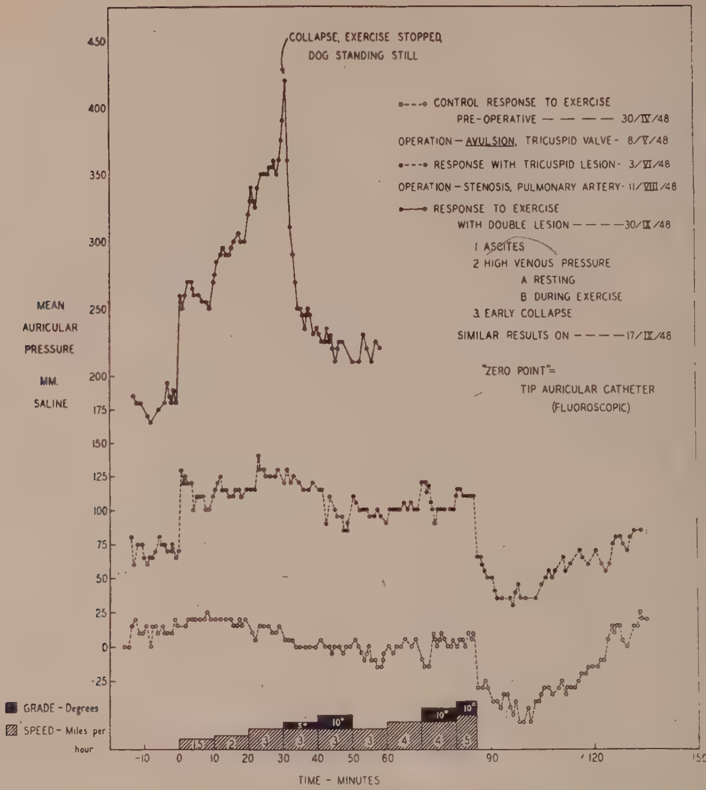

FIG. 1.

Chart showing representative changes in right auricular pressure (mm saline) during exhausting exercise. (Dog No. 2.)
A. (lower curve) Normal dog (preoperative).
B. (middle curve) Same dog following tricuspid valve avulsion.
C. (top curve) Same dog following subsequent pulmonary stenosis.

right ventricular myocardium, and a small incision made. A curved valvulotome was inserted through the incision, and the leaflets of the tricuspid valve were cut blindly. The myocardium was then closed with a mattress suture. In one dog (No. 1) a more satisfactory approach was made through the 4th interspace on the right. Purse-string sutures were placed on the right auricular appendage, and an incision made for the passage of the valvulotome. The instrument was introduced into the right ventricle through the auriculoventricular orifice and the cusps of the tricuspid valve avulsed. The animals were up and active 8 to 12 hours after operation and had uneventful recoveries.

After operation the dogs were allowed a 3-week period of recovery before a postoperative experiment was performed on the treadmill under the same conditions as the control run. After this the dogs were given daily bouts of strenuous exercise for 3 months or more, and at least one more standard treadmill experiment performed before the second operation.

The main pulmonary artery was stenosed according to the method of Hufnagel *et al.*(6) The left thorax was entered through the 4th interspace. The pulmonary artery was dissected free and a special clamp placed on the artery occluding the desired amount of the vessel (approximately 50% of the diameter in these operations). A running mattress suture was then placed along the proximal

TABLE I.
Mean Right Auricular Pressure and Heart Rate in Normal Dogs (A), and in the Same Dogs Following Tricuspid Valve Avulsion (B), and Superimposed Pulmonary Artery Stenosis (C).

	Mean right auricular pressure (mm saline)			Heart rate per min.		
Dog	Standing at rest on treadmill	Onset of exercise	At exhaustion	Standing at rest on treadmill	Max. heart rate	Reduction in work tolerance
			A. Control.			
1.	0	—10	0	85	?	0
2.	15	20	10	115	295	0
3.	0	50	—60	100	215	0
		B. Tricuspid valve avulsion.*				
1.	125	160	230	160	250	0
2.	70	125	110	125	275	0
3.	100	135	190	125	280	0
		C. Pulmonary stenosis (50% of diameter occluded).				
1.	225	310	310†	185	195	marked
2.	180	260	420	180	195	moderate
3.	175	215	335	130	250	moderate

* The extent of valvular damage determined at autopsy was: Dog No. 1, 2 cusps destroyed, 3rd damaged; Dog No. 2, 1 cusp destroyed, 1 damaged; Dog No. 3, 3 cusps damaged only.

† Collapsed after 2 min. at 2 M.P.H. on the level.

edge of the clamp. The segment of the wall thus separated from the lumen of the pulmonary artery was then resected, leaving a tapered area of stenosis. Frequent extrasystoles were noted for the first 24 to 48 hours postoperatively, but recovery was uneventful otherwise.

Observations. In the normal dog, as shown in the lowest curve of Fig. 1, auricular pressure remained constant or decreased slightly during periods of increasing exercise, with a sharp drop immediately after exercise. Following tricuspid avulsion right auricular pressure was moderately elevated at rest (Fig. 1, middle curve), rose further with the onset of exercise, but otherwise the changes in auricular pressure were similar to those of the control experiment in the example illustrated, though at a higher level throughout. The mean auricular pressure in the other 2 dogs rose 55 to 70 mm above that at the onset of exercise (Table I). None of the dogs at this stage had any detectable reduction in work tolerance, nor did any other evidence of failure appear, despite daily bouts of exhausting exercise for 3 months or more.

When the dogs with insufficiency of the tricuspid valve were subjected to stenosis of the pulmonary artery in addition, a further rise of resting auricular pressure occurred (Fig. 1, top curve and Table I.) With the onset of the mildest grade of exercise auricular pressure rose considerably, and then rose to still higher levels with each added increment of work, reaching the remarkable height of 420 mm saline before the animal collapsed at the relatively light work load of 3 miles per hour and 5° grade. Heart rate was rapid before exercise, and only rose 15 beats a minute during exercise (Table I). Similar results were obtained in the other 2 dogs (Table I).

All 3 dogs with tricuspid regurgitation and pulmonary stenosis developed a syndrome similar to chronic, congestive failure and characterized by markedly elevated auricular pressures and distended veins, decreased work tolerance, hepatomegaly, ascites, tachycardia at rest, and a relatively fixed heart rate during exercise. The rate at which cardiac failure progressed varied from animal to animal. Ascites was noted in dog No. 1 within 2 weeks of pulmonic stenosis, and while he was still restricted to his cage. His work tolerance was extremely limited, and he collapsed after 2 minutes at 2 miles per hour on the level. Dog No. 2 developed ascites one month postoperatively, after several days of mild exercise. Dog No. 3, whose work tolerance was reduced less than the other dogs postoperatively, did not develop ascites until 6 weeks after operation, and then only after daily exhausting exercise.

For additional evidence that the syndrome was due to cardiac insufficiency, 2 of the dogs were sacrificed for study in heart-lung preparations. Cardiac output was low for both hearts. To obtain cardiac outputs approaching the normal range for fresh heart-lung preparations required extremely high auricular pressure, *e.g.*, 150 to 200 mm of saline. Calculation of the "competence index"(7) also indicated marked cardiac impairment.

The animals were sacrificed 1½ to 3 months after pulmonary stenosis was produced. Autopsies revealed dilatation and hypertrophy of the right auricle and ventricle in each dog. The pleural cavity was dry and the lungs normal. The liver was markedly enlarged and congested, and 2 to 5 liters of serosanguinous fluid were found in the peritoneal cavity.

Summary. A form of progressive, chronic, "right-sided" cardiac failure has been produced in dogs by tricuspid valve avulsion and pulmonary artery stenosis. Three dogs so treated developed congestive failure with elevated auricular pressure and distended veins, decreased work tolerance, hepatomegaly, ascites, tachycardia at rest, and a relatively fixed heart rate during exercise. Studies of 2 of the hearts in the heart-lung preparation indicate clearly that cardiac insufficiency was present.

We are indebted to Dr. Eugene M. Landis for suggesting this problem, and for his advice during the work. It is also a pleasure to thank Dr. Otto Krayer for performing the heart-lung experiments, and Dr. Charles A. Hufnagel for surgical advice and assistance.

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Thyroid Function in the Alarm Reaction. (17596)

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The response of the body to stress of various kinds has received considerable attention in recent years; the majority of investigations have been concerned with the role of the pituitary-adrenocortical system under such conditions. It appeared *a priori* probable that the pituitary-adrenocortical system would not be the only one involved. Some rather specific forms of stress, "alarming stimuli", are quite well known to involve also the pituitary-thyroid system, *e.g.* reduced oxygen pressure (high altitude), and changes of environmental temperature. In an interesting and stimulating speculation, Means(1) considered the possibility that Graves' disease might in some way be related to an alarm reaction, a thought which he discusses in the first edition of his monograph. However, this speculation appears to have lost its appeal to its author, because it is omitted from the second edition. We decided to investigate the response of the thyroid to alarming stimuli. While our work was in progress, two reports on this subject appeared,(2,3) indicating the general interest in the complicated response to stress on the one hand, and in the adrenocortical-pituitary-thyroid interrelationship on the other hand.

Materials and methods. A total of 106 adult male rats was used in this study. Animals were kept on a stock diet of Purina dog chow. Intramuscular injection of 10% formalin was used as the stress or alarming stimulus. Thyroid function was gauged by uptake of tracer doses of I-131.* The latter

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TABLE I.
Uptake of I-131 and Thyroid Weight Following Formalin Injection.

	Thyroid: counts/mg/sec.				
Exp. No.	Controls	Formalin (10%)	Diff.	"p" of Diff.	Thyroid wt (mg/100 g body wt) % decrease
1	55.3 (3)*	36.2 (3)†	19.1	<.4	18
2	145.7 (5)	54.9 (5)‡	90.8	<.01	32
3	137.4 (5)	70.2 (6)§	67.2	.01	17
4	59.4 (10)	33.9 (10)‡	25.5	<.001	21
5	78.1 (10)	63.4 (10)‖	14.7	<.01	36

* Fig. in parentheses = No. of rats.
† 1 inj. of 2 cc, tracer 48 hr later (4 μc).
‡ 4 inj., .5 cc each, in 2 days, tracer 24 hr after last inj. (4 μc).
§ 3 inj., .5 cc each, in 2 days, tracer 24 hr after last inj. (4 μc).
‖ 1 inj., .5 cc, tracer 17 hr later (4 μc).

TABLE II.
Uptake of I-131 Following Injection of Cortical Hormone.

		Thyroid: counts/mg/sec.		
Exp.	Treatment	Treated	Controls	Difference ("p" of Diff.)
6	D.O.C.A., 2 days* 40 mg/day	69.7 (9)‡	59.4 (10)	10.3 (<.1)
7	Aqu. Adr. Extr.† (Upjohn) 1 cc every hr. 7 inject.	39.8 (9)	29.5 (8)	10.3 (<.1)

* 4 μc I-131 given 24 hr after last inj. of D.O.C.A.
† 4 μc I-131 given 1 hr after last inj. of Adr. Cort. Extr.
‡ Figures in parentheses indicate number of rats.

was administered by intraperitoneal injection as sodium iodide without carrier. Rats were killed four hours after injection of I-131; the thyroid glands were dissected out, weighed rapidly on a Roller-Smith Torsion balance, and were immersed in 2% sodium hydroxide solution. Aliquots were taken for determining radioactivity by means of a Geiger-Muller Counter. Because the application of an alarming stimulus is followed by increased secretion of adrenocortical hormone, an attempt was made to duplicate results obtained with injection of formalin by injection of adrenocortical hormone. No crystalline C-11 oxygenated compound was available to us. Aqueous adrenocortical extract (Upjohn) was employed, assayed by us to contain approximately 350 μg equivalents of compound A acetate. In another experiment desoxycorticosterone acetate was used.

Results. Results are summarized in Tables I and II. As can be seen from Table I, the uptake of I-131 was decreased in every instance following injection of formalin; this decrease was statistically significant in all but one experiment; in the latter only 3 rats were used, and a large dose of formalin given in a single injection had proven very toxic rendering the animals exceedingly sick at the time the tracer dose was administered. The weight of the thyroid gland (mg per 100 g body weight) was decreased in all experiments.

As can be seen from Table II, the uptake of I-131 was increased slightly following treatment with desoxycorticosterone acetate and with aqueous adrenocortical extract, but the increase is statistically not significant.

Discussion. Before discussing the results obtained, two points in our procedure require brief mention.

Diet. Many authors, using the rat in tracer studies with I-131, have employed a diet slightly deficient in iodine (Steenbock diet with added potassium iodide), as suggested by

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* Radioactive Iodine (I-131) was supplied by the Atomic Energy Commission, Oak Ridge, Tenn.

Rawson *et al.*(4) The latter authors chose this diet in order that animals might not be protected by iodine against the effects of thiocyanate. At the same time, the uptake of I-131 by the rats' thyroid is increased under this iodine deficient regime. At least within certain limits, the uptake of I-131 is inversely proportional to the amount of potassium iodide added to the stock diet.(5) Whereas use of the iodine-poor diet offers the technical advantage of increased uptake, it introduces a further metabolic variation, the significance of which for the problem under study could not be foretold. For this reason we have used a laboratory stock diet and tap water.

Calculations of Uptake. It is customary to report the uptake of I-131 by thyroid glands, and to compare the total uptake by control animals with that obtained under conditions of an experiment. It appears to us that this is a valid indication of altered thyroid function only if the experimental procedure does not involve a change of thyroid weight. Otherwise the possibility exists that increased or decreased uptake of the tracer might be due to changes in the amount of functioning tissue only, without necessarily indicating alteration in function. We have therefore calculated the uptake per milligram of thyroid tissue, expressed in the table as counts per milligram per second.

Thyroid Function Under Conditions of Stress. The observations presented in this paper indicate that thyroid function is depressed in a condition of acute stress (formalin injection). This finding is similar to that reported by Soffer *et al.*(3) who found decreased uptake of I-131 following injection of epinephrine in the intact animal; however, epinephrine increased the uptake in adrenalectomized rats and this increase could be prevented by treating the adrenalectomized rat with compound E. The authors concluded that the adrenal cortex exerts an inhibitory effect on the thyrotrophin-stimulating effect of epinephrine. In our formalin-treated rats the thyroid weight was decreased, confirming Selye's statement(6) that thyroid "atrophy" is observed early during the alarm reaction. The finding of decreased thyroid function under conditions of stress (formalin injection: present authors; Epinephrine: Soffer *et al.*)(3) is at variance with observations in humans reported by Reiss *et al.*(2) who found increased uptake of I-131 following epinephrine injection in normal individuals and following injection of compound E in Addison patients.

At the present time little can be gained by attempting to explain these apparent discrepancies, which might conceiveably be due to species differences, to differences of timing or to a number of unknown factors. Both in Soffer's and in Reiss's observations, 11-dehydro-17-hydroxycorticosterone (Compound E) influenced the uptake of I-131 by the thyroid, although the effect was in opposing directions. In our experiments, desoxycorticosterone acetate (40 mg daily) failed to influence significantly uptake of I-131 by the rat's thyroid, nor did it have any effect upon inhibition of thyroid function induced by stress. No influence of aqueous adrenocortical extract (Upjohn) upon uptake of I-131 was observed in the intact rat.

Summary. Rats under the influence of an alarming stimulus (formalin injection) showed decrease in thyroid function, the latter evidenced by diminished uptake of I-131 per milligram of thyroid tissue. Desoxycorticosterone acetate and adrenal cortical extract failed to influence thyroid uptake of I-131 significantly.

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Visualization of Tobacco Mosaic Virus Within Infected Cells. (17597)

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One of the most interesting problems arising within our present ability to see elementary virus particles deals with their development from the living cells they infect. Much knowledge concerning this has already been gained through the electron microscopic examination of bacteriophages and their bacterial hosts which, growing as separate entities, are accessible to thorough electron microscopic inspection. A broadened attack on this problem of virus proliferation is, however, now being made possible by the development of suitable ways of cutting tissue sections thin enough for study under the electron microscope. We have been using these methods to investigate how certain viruses develop within diseased plants; this note records preliminary observations and interpretations on about 200 photographs made on the occurrence of tobacco mosaic virus (*Marmor tabaci* H.)(1) in leaf cells of diseased Turkish tobacco (*Nicotiana tabacum* L.).

For the electron micrographs shown here, small pieces were cut from leaves showing marked symptoms of systemic infection with tobacco mosaic virus. These bits, most of them taken from leaf margins, and similar pieces cut from healthy Turkish tobacco plants of corresponding age were immediately fixed by immersion in the conventional formalin-aceto-alcohol mixture. When ready for sectioning they were removed from this fixative, washed thoroughly, passed through the usual alcohols, embedded in methacrylate, cut and prepared for electron microscopy by the procedures recently described by Neuman, Borysko and Swerdlow.(2) The finished sections after removal of methacrylate were shadowed with gold-manganin and photographed in RCA electron microscopes. This shadowing contributes little to the visibility of fine structure in such sections but serves other useful purposes. It prevents the accumulation of charge on the thicker portions of the section, with the disturbing effects such charges have on the quality of the electron image, it permits ready distinction between detail lying deep in the section and on its surface and it prevents the serious distortion of the section that otherwise commonly occurs when an electron beam strikes it.

Typical sections through a healthy Turkish tobacco leaf show chloroplasts lining more or less completely the well-defined walls of its cells (Fig. 1). As this figure indicates, most chloroplasts have one or more large opaque inclusions, the starch grains. However, holes are usually seen where these objects have

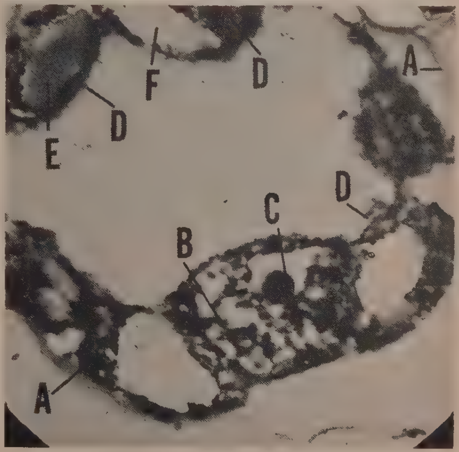

Fig. 1.

A cross section through a cell of a healthy leaf of Turkish tobacco showing at (A) cell wall; (B) interior of a nucleus; (C) nucleolus; (D) chloroplasts; (E) starch grain; (F) cavity from which starch grain was knocked out. Magnification = 8800 ×.

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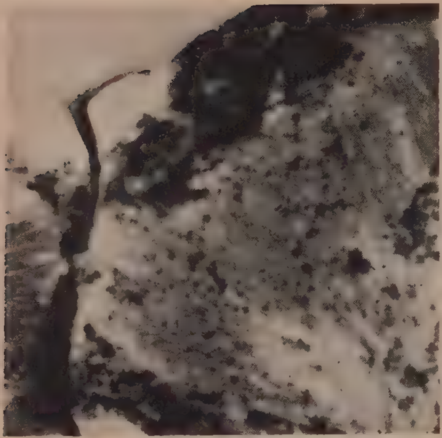

FIG. 2.
Section through parts of two cells of a tobacco leaf diseased with tobacco mosaic virus. Some of the finer filaments in the right hand cell (which occupies most of the figure) have the thickness to be expected of single tobacco mosaic rods, though their lengths are many times 2800A. 8800 ×.

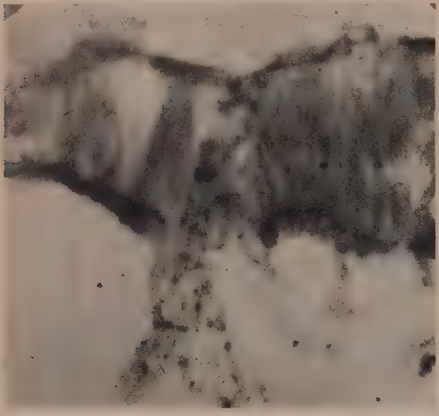

FIG. 3.
A mass of virus with filaments and strands extending into the region of the cell vacuole. 8000 ×.

been punched out during the sectioning. The cell nuclei which occasionally are sectioned appear as coarse, especially opaque networks having a well marked membrane and often a very opaque nucleolus.

Sections through diseased leaves show some cells of normal appearance, but most cells contain visible amounts of virus in the form of masses of strands or fibers. In Fig. 2 and 3 some of these fibers appear as separate filaments having approximately the 150A diameters typical of tobacco mosaic virus rods. It is important that these separate filaments, where they have been seen clearly, have nearly always been many times longer than the 2800A particles that predominate in suspensions freshly made from diseased tissue.(3) Evidently in the plant these relatively short elementary particles are usually associated

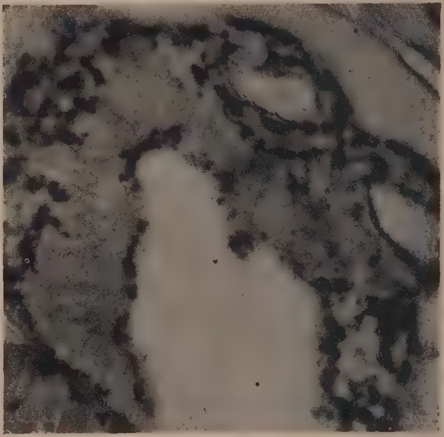

FIG. 4.
Virus filaments radiating inwards from a cell wall and the chloroplasts that line it. 8000 ×.

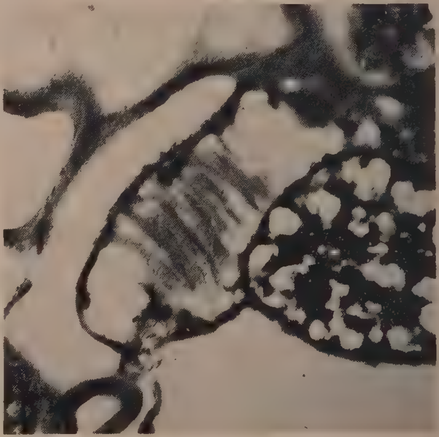

FIG. 5.
Body with well-defined membrane containing virus. The adjacent nucleus appears normal and virus-free. 9300 ×.

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together end-to-end as well as side-to-side. In Fig. 2 the virus appears to fill much of the cytoplasmic region of the diseased cell; it has reached deeply into the central vacuole in the cell of Fig. 4. In the fully diseased leaf tissue described here, the virus has usually occurred in thick fibrous masses (Fig. 6 and 8) rather than as separate, individual particles. In all cases the fibrous strands seem to extend inwards from the cell wall (Fig. 2 and 4).

Many of the virus masses are in close association with the chloroplasts and even appear to be internal to them. A cell body containing virus is shown in Fig. 5. This body has a well-defined membrane, but it is not certain whether it is a chloroplast or an inclusion body. The elongated body of Fig. 7 containing a fibrous mass of virus has a hole like that left in chloroplasts when the starch grain is dislodged. Virus masses are often seen extending out from what appear to be damaged chloroplasts (Fig. 2, 4, 6 and 8). Whether such disintegration of the chloroplasts is due to virus action alone or to virus plus the treatment of the material in preparation for electron microscopy is of course not known. It has not been encountered in healthy cells. The inner edges of the virus masses of Fig. 4, 6 and 8 carry lines of dark granules that may well be fragments of broken chloroplasts. Unlike the chloroplasts from some other plants we have examined, the inner detail of those from both healthy and diseased Turkish tobacco has not been sufficiently well defined in section to identify these granules with the grana.

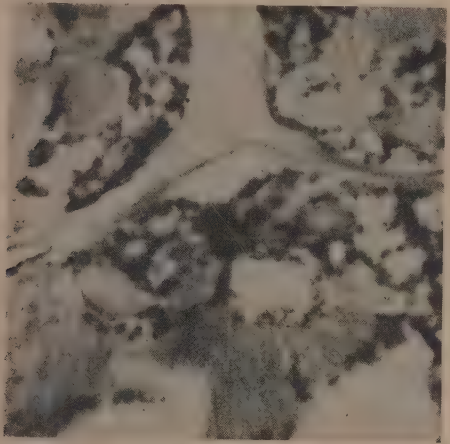
FIG. 6.
Much virus appears in the bottom cell of the three that meet at the center of the photograph; less virus can be seen in the other two. 8000 ×.

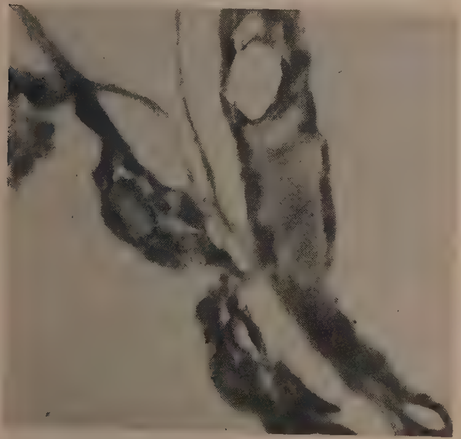
FIG. 7.
A mass of virus within a body that may have been a chloroplast. 8800 ×

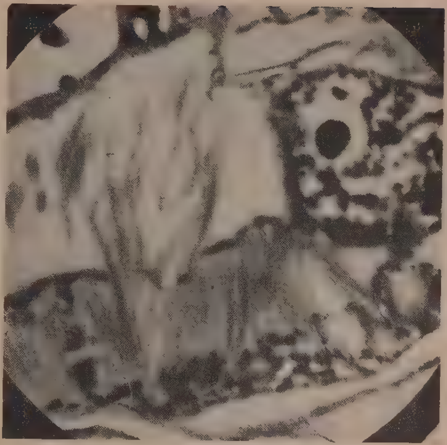
FIG. 8.
Virus dispersed into the region of the central vacuole of a cell from the mass at the bottom. The apparently normal nucleus is at the right. 8800 ×.

In contrast with the marked changes in the aspect of the chloroplasts, the nuclei appear the same in healthy and diseased cells and show no evidence that they contain strands of virus (Fig. 1, 5 and 8).

This work is being continued with plant

tissues diseased with tobacco mosaic and with other viruses to see what further information can thus be gained concerning the focus and mechanism of virus proliferation.

Summary. Electron micrographs of sections cut through tobacco leaves infected with tobacco mosaic indicate that the virus is often present in fibrous masses associated with chloroplasts which are severely affected. Virus can clearly be seen in the cytoplasm of the diseased cells sometimes as single filaments. The nuclei seem unaltered by the infective process and have appeared free of virus. The great length of these filaments, much in excess of 2800A, indicates an end-to-end arrangement of the rods *in situ.*

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Increased *in vitro* Tuberculin Leucocyte Cytolysis Following a Tuberculin Skin Test.* (17598)

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(Introduced by J. H. Mueller)

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It has recently been shown that leucocyte suspensions prepared from the blood of animals showing tuberculin hypersensitivity are injured when exposed to tuberculin.(1,2) Further analysis of this cell-tuberculin-system has shown that a serum factor, present in the euglobulin portion of serum from tuberculous subjects,(3,4) is capable of destroying portions of the white cells of the tuberculous as well as the non-tuberculous guinea pig and man, if first these cells are exposed to tuberculin.(5) A convenient way of demonstrating this serum factor consists in following the white blood counts in samples of heparinized whole blood to which appropriate amounts of tuberculin have been added.(6)

The purpose of this report is to describe an increase in *in vitro* tuberculin-leucocyte cytolysis in blood of tuberculin sensitive guinea pigs following an intracutaneous tuberculin reaction. This study was prompted by earlier findings that a positive tuberculin test in man may be followed by a similar increase in *in vitro* tuberculin cytolysis.(6)

Materials and Method.

Materials. Guinea Pigs. Four hundred to 900 g animals were inoculated one to 3 times at 2-month intervals in both groins and pectoral regions with 10 mg of heat-killed tubercle bacilli (H37Rv) suspended in 1 ml of Bayol F (0.25 ml at each site). Animals were selected for study who showed hard masses 1 to 2 cm in diameter in the above areas and who also demonstrated cutaneous induration greater than 1 cm in diameter at 48 hours from a second strength PPD (5 γ in 0.1 cc) skin test.(7) Normal tuberculin-negative guinea pigs were used as controls. No skin tests were done more recently than 3 weeks before the experiment began.

Leucocytes. Two ml blood samples were taken by cardiac puncture into a syringe containing 0.2 ml heparin (Liquaemin), mixed,

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* Work done under an U. S. P. H. S. Research Grant.

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TABLE I.
In vitro Effect of PPD upon the Leucocyte Count on Animals Before and After Receiving an Intracutaneous Tuberculin Test. Animals 1 through 8 are experimental; Animals 9 through 16 are controls.

Before PPD Skin Test. Date 7/19.					After PPD Skin Test. Date 7/21.				
G.P. No.	Avg WBC	% fall Saline	% fall PPD	PPD decrement	Skin test at 48 hr, mm	Avg WBC	% fall Saline	% fall PPD	PPD decrement
1	4760	+ 0.5	—14.4	—14.9	12x13	7680	0	—28.8	—28.8
2	5720	— 1.1	— 1.1	0	10x10	10630	—0.2	—23.7	—23.5
3	8870	— 0.6	— 9.7	— 9.1	11x11	12700	—0.6	—17.6	—17.0
4	6350	—27.3	—24.8	+ 2.5	12x12	11330	—0.5	—25.8	—25.3
5	8980	— 0.3	—13.3	—13.0	11x11	10780	0	—19.6	—19.6
6	6420	+ 0.5	— 1.6	— 2.1	18x16	8370	—1.0	—29.1	—28.1
7	4850	— 0.3	—13.4	—13.1	20x15	7260	+0.8	— 8.4	— 9.2
8	4920	— 0.7	+ 1.0	+ 0.3	17x16	12690	—0.2	—24.5	—24.3
9	3650	— 0.5	— 3.3	— 2.8	neg.	3810	+0.6	— 1.5	— 2.1
10	3640	+ 1.7	0	— 1.7	,,	6250	—1.3	— 0.7	+ 0.6
11	6190	+ 0.3	+ 0.2	— 0.1	,,	4710	—1.5	— 1.5	0
12	4940	— 1.1	+ 0.6	+ 1.7	,,	4950	+0.5	— 0.3	— 0.8
13	2300	— 1.0	+ 0.5	— 0.5	,,	3330	—0.2	+ 0.5	+ 0.7
14	3650	— 0.7	— 1.2	— 0.5	,,	4380	—1.0	— 1.3	— 0.3
15	10300	+ 0.6	+ 0.1	+ 0.1	,,	11170	0	0	0
16	7070	+ 0.3	+ 0.5	+ 0.2	,,	8760	—0.7	— 0.5	+ 0.2

PPD—5 γ/0.1 cc—planted intracut. 0.1 cc per guinea pig.

and the white cells counted by the method described elsewhere.(5,6)

Tuberculin. PPD used was freshly prepared by dissolving one tablet of commercial second strength PPD(7) in 0.5 ml of 0.85% saline and using 0.1 ml for the test. This resulted in a concentration of 50 γ per ml of cell suspension. Old Tuberculin used was obtained from the Massachusetts Department of Health. The crude O.T. was dialized against 3 changes of 0.85% saline and used as a 1:10 dilution or a concentration of 1.5 mg per ml of cell suspension.

Method. To 0.4 ml heparinized blood in each of 2 siliconed Wassermann tubes was added 0.1 ml of PPD solution and 0.1 ml of 0.85% saline respectively. After adequate mixing leucocyte counts were performed (5-minute counts). The tubes were then rolled at 100 RPM in an incubator for 1 hour before the second leucocyte count. The PPD decrement is the per cent fall in the tuberculin tube minus the per cent fall in the saline control tube.

Results. In Table I are given the cytolysis tests on a group of 8 tuberculin-sensitive and 8 normal guinea pigs which were studied just before and 48 hours after receiving 5 γ of PPD intracutaneously. This dose resulted in a typical indurated reaction 1 to 2 cm in diameter in all experimental animals. Inspection of Table I shows that 7 out of 8 experimental and no one of the control animals showed a greater decrement following the cutaneous tuberculin reaction. In a subsequent experiment essentially similar results were obtained on blood samples from the same animals using O.T. as a skin testing antigen. Animal 4 in Table I was bled with some difficulty and experienced a mild "alarm reaction."(6) The size of the skin test did not correlate with the cytolysis test.

Discussion. In an attempt to transfer passively tuberculin hypersensitivity to normal rabbits by the injection of whole blood from tuberculous rabbits, Sandage and Birkeland(8) noted that this could be done successfully only when the blood was used from animals during their response to an intravenous injection of tuberculin. Observations reported here indicate that a recent tuberculin reaction alters *in vitro* tuberculin cytotoxic responses as well. Accordingly, passive transfer of the tuberculin reaction with whole blood is a more complex event than that with washed cells using the method of Chase.(9)

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Summary. Guinea pigs which have been sensitized to tuberculin by the injection of heat-killed tubercle bacilli in paraffin oil show an increased *in vitro* tuberculin leucocyte cytolysis within 48 hours after a positive intracutaneous tuberculin reaction.

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Appearance of Protein Tagged with Radioactive Iodine in Thoracic Duct Lymph.* (17599)

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When plasma protein or albumin is tagged with radioactive iodine (I-131) and the combination is injected intravenously into dogs or humans to determine the plasma volume, there is a progressive decrease in the measurable radioactivity of blood samples withdrawn periodically.(1,2) This suggests the passage of the iodinated protein into extravascular spaces. It is known that protein circulating in the blood stream passes with considerable facility into the thoracic duct lymph. The concentration of protein in lymph varies in different parts of the body. Field *et al.*(3) reported the plasma protein concentration in 13 dogs to be 6.25 g per 100 cc whereas the concentration in thoracic duct lymph was 4.00 g per 100 cc. The average concentration of albumin in the plasma and thoracic duct lymph of the same animals was 3.61 and 2.45 g per 100 cc respectively. The purpose of the present experiments was to determine the rapidity with which the intravenously injected radioactive protein appeared in thoracic duct lymph. The amount of radioactivity in lymph 10 minutes following the intravenous injection was of special interest, since this period is the commonly accepted interval between the injection of either radioactive protein or T 1824 and withdrawal of blood for the determination of this plasma volume.

Method. Eleven healthy adult fasting dogs, weighing 8 to 16 kilos, were used. Each dog was anesthetized with intravenous sodium pentobarbital .030 g per kilo of body weight. Through a left cervical approach the junction of the thoracic duct and the left subclavian vein was exposed. The duct was ligated close to the subclavian vein and, depending upon its size, a number 20 or 18 gauge needle was inserted into the lumen of the duct and fixed with a No. 000 black silk ligature. The needle was irrigated with a few drops of heparin to prevent clotting. A femoral vein and artery were exposed and isolated to be used for injection of the iodinated protein and collection of blood samples. Twenty cc of iodinated protein containing less than 1 g of albumin and approximately 40 microcuries of radioactivity were injected into the femoral vein. Simultaneous samples of blood and lymph were collected from the femoral artery and thoracic duct 10, 30, 60, and 120 minutes following the injection. The animals were divided into two groups. Group I consisted of 6 dogs into which radioactive iodinated human albumin was injected. Group II consisted of 5 dogs into which was injected canine plasma protein activated with the iodine. Technical difficulties prevented the collection of some of the lymph samples in the first

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The authors wish to acknowledge the technical assistance of Ruth Stavitsky and Jean E. Hower.

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TABLE I.
Percent Radioactivity of the Thoracic Duct Lymph in Animals Injected with Human Albumin Tagged with Radioactive Iodine.

	Radioactivity $\frac{\text{cc/chyle}}{\text{cc/plasma}} \times 100$ Time after injection			
Dog No.	10 min.	30 min.	60 min.	120 min.
6	9.6	33.9		
8	4.6	20.2		
9	3.2	36.8	53.9	
10	10.6	49.1		53.8
11	4.0	10.1		44.6
12	8.9	34.1		59.6
Avg	6.8	30.7		52.7

TABLE II.
Percent Radioactivity of the Thoracic Duct Lymph in Animals Injected with Canine Plasma Protein Tagged with Radioactive Iodine.

	Radioactivity $\frac{\text{cc/chyle}}{\text{cc/plasma}} \times 100$ Time after injection			
Dog No.	10 min.	30 min.	60 min.	120 min.
13	1.5	6.2	19.9	35.5
14	2.5	4.8	15.0	29.7
15	1.7	12.0	31.6	43.0
16	0.7	15.5	38.6	54.4
17	0.3	9.6	24.1	36.8
Avg	1.3	9.6	25.8	39.9

group. Lymph samples were collected by allowing the lymph to drop into a test tube containing a few crystals of powdered heparin. Blood samples were collected from the femoral artery. The blood was centrifuged for 15 minutes at 3,500 rpm and the supernatant plasma decanted. One cc samples of lymph and plasma were pipetted into small porcelain dishes and dried at room temperature for 24 hours. The radioactivity of the samples was then determined by means of a Geiger-Mueller counter. The radioactivity of a sample of lymph was compared to that of a corresponding plasma sample as follows:

$$\frac{\text{Counts per min. per cc of lymph}}{\text{Counts per min. per cc of plasma}} \times 100 = \%\ \text{radioactivity of lymph}$$

Results. Table *I* shows the per cent radioactivity of the lymph samples of each dog in Group I. There is a progressive increase with the time the sample was obtained. There is some variation in the values ranging from 3.2% to 10.6% for the 10-minute specimens and from 10.1% to 49.1% for the 30-minute specimens. Insufficient 60- and 120-minute samples were obtained in this group to warrant a comparison of results.

The per cent radioactivity of the lymph samples of Group II are shown in Table II. This series demonstrates some variation in values between the animals but of a lesser degree than was noted in the previous group. Values ranging from 0.3% to 2.5% were found in the 10-minute specimens and values ranging from 4.8% to 15.5% were encountered in the 30-minute specimens. It is noted that the average value for the 10-minute specimen in Group I was 6.8% whereas in Group II it was 1.3%. Similarly the average percentage for the 30-minute specimen in Group I was 30.7% compared to 9.6% in the second group. There is little doubt that the human albumin diffused from capillaries into lymphatic spaces much more rapidly than did the canine serum protein. It is possible that the use of a foreign protein (human albumin) produced an alteration in capillary permeability and allowed the more rapid passage of the protein into the thoracic lymph. Also, in the first group of animals, manual pressure was applied to the abdomen in order to increase the flow of lymph; this was not true in the second group. The trauma associated with this procedure may have produced some increase in capillary permeability.

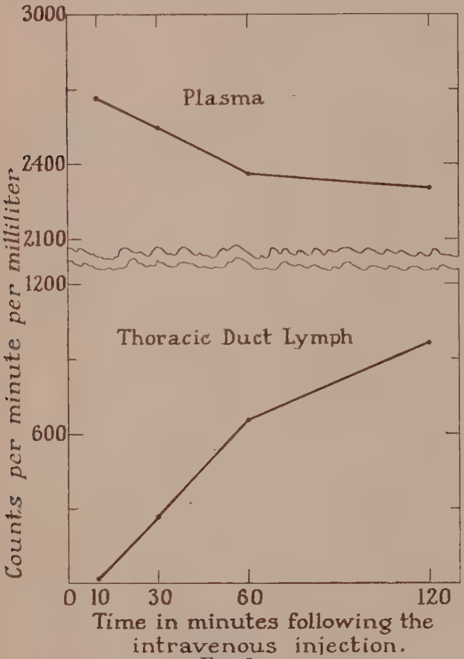

FIG. 1.

Illustration of the increase and decrease of radioactivity of the thoracic duct lymph and plasma respectively, during the 2-hour interval following the intravenous injection of radioactive iodinated protein. The curves represent averages from the 5 dogs in Group II.

Fig. 1 demonstrates the changes in concentration of radioactive iodinated protein in the thoracic duct lymph and the plasma plotted against time. During the first hour, there is a rapid increase in the concentration of the protein in the lymph followed by a more gradual increase. There is a simultaneous decrease in the concentration of the protein in the plasma. Cope and Moore demonstrated that the radioactivity of the serum and thoracic duct lymph reach an equilibrium between six and seven hours following the intravenous injection of radioactive protein.(4) Amino acids are present in thoracic duct lymph. Petersen and Hughes found an average of 23.9 mg per 100 cc in 28 dogs.(5) Lymph samples from two dogs in Group II were dialyzed in a cellophane bag for 48 hours in order to remove any free amino acids or electrolytes which were present. There was no change in the activity of this lymph, indicating that all the radioactivity was due to albumin-bound I-131.

Conclusions. 1. The total lymph volume is not measurable, so that the exact amount of activated protein that is present in the body lymph, following intravenous injection of iodinated protein, cannot be calculated. However, it appears from this experiment that a negligible amount is present in the thoracic duct lymph 10 minutes after injection.

2. During the first hour there is a rapid increase in the lymph content of radioactive protein followed by a more gradual rise.

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Effect of Biotin Deficiency Upon the Skin of Mice. (17600)

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Wilson *et al.*(1) have shown that nutritional biotin deficiency in the mouse, is expressed in severe dermatotic symptoms which are superficially similar to those described by Sullivan and Nicholls(2) in the rat. This paper reports the histological changes in the skin of biotin-deficient mice. Several of these details, and particularly the changes in the sebaceous glands, and the behavior of connective tissue cells in the dermis have not been reported hitherto either in the rat or the mouse.

Methods. The animals used in this study are those described by Wilson *et al.*(1) and Wilson and Leduc(3) in a study of biotin deficiency in the mouse. The procedures for rendering the animals deficient in biotin are described in detail by these authors. The 50 animals used here were chosen to represent several degrees of symptomatic deficiency. Those animals which will be referred to as very deficient showed extreme symptoms and were nearly moribund. Skin was removed from the mid-dorsum and from the perineal region, including the whole anal canal. The perineal skin was particularly useful in the study of sebaceous glands which are large and numerous in this region. Tissues fixed in Zenker-formol were stained in Delafield's hematoxylin and eosin, and in Heidenhain's hematoxylin. Other sections were stained in eosin-methylene blue to reveal cytoplasmic basophilia, and with toluidin blue for the study of metachromasia (see Lison,(4) for evaluation and manipulation of this technic).

The lipids were demonstrated in frozen sections of tissues fixed in formol-calcium and then chromated in dichromate-calcium of Baker(5). Sections were treated with Sudan black and with oil-blue N. It has become increasingly evident that the lipids are much more clearly discerned in tissues thus treated than in tissues simply fixed in formol-calcium. For the demonstration of delicate lipid granules, Sudan black is superior to oil-blue N. Unstained frozen sections were studied under the phase-contrast microscope for the fine morphology, and under polarized light for birefringence. Representative sections were treated with the Romieu test for cholesterol and its esters (from Lison)(6), after being oxidized for 3 days in 2.5% ferric alum at 37°C. In all of these studies, the skin of experimental mice is compared with that of normal control animals.*

Results. The slightly thicker than normal epidermis of moderately deficient mice is usually keratotic, and contains numerous keratohyalin granules. Mitotic figures in the lower layers of the stratum spinosum and in the basal layer appear slightly more numerous than in normal skin. Hair follicles are normal, but in the sebaceous glands some of the peripheral cells are fragmented. The non-sebaceous epithelial elements associated with the sebaceous glands are thicker than in normal glands. There is some mitotic activity in the non-sebaceous elements and in the cells at the periphery of the glands. Histiocytes, mast-cells, eosinophiles and other leucocytes, in the dermis appear to have increased in number.

In very deficient mice, all of the changes mentioned above are accentuated. The stratum corneum and the whole epidermis are thicker and the keratohyalin granules in the upper cells of the stratum spinosum are more numerous. In some animals conspicuous epi-

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* I am indebted to my colleague, Professor J. W. Wilson, and to Dr. E. H. Leduc for providing me with all of the material used in this work.

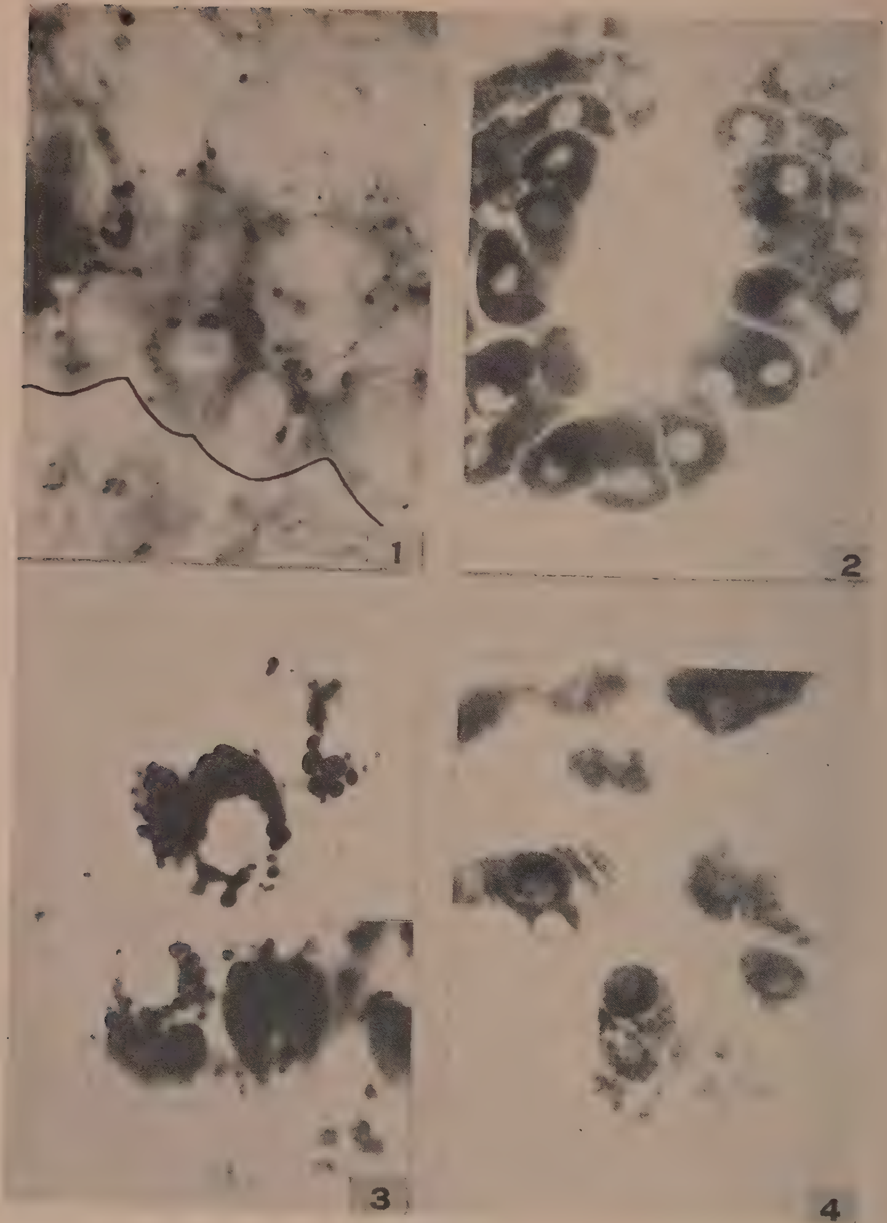

PLATE 1.

All of the frozen sections figured are cut at 5 μ and colored with Sudan black.

FIG. 1. Skin of a biotin-deficient mouse. Inked line separates epidermis from dermis. The coarser lipid bodies in the epithelial cells resemble Golgi elements. The finer lipid bodies, not clearly shown, surround the nuclei. 1125 ×.

FIG. 2. Sebaceous gland of skin of a normal mouse. Note especially the fine lipid granules in sebaceous cells. 562.5 ×.

FIG. 3. Details of sebaceous glands of a very deficient animal. Compare with Fig. 2. 675 ×.

FIG. 4. Fragmenting sebaceous gland of a very deficient mouse. 562.5 ×.

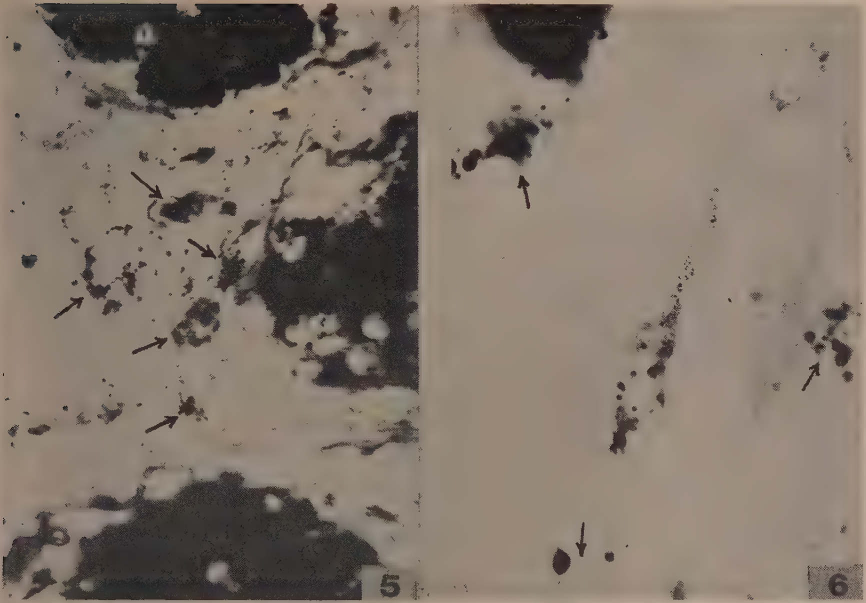

PLATE 2.

FIG. 5. Macrophages, laden with lipids, surrounding fragmenting sebaceous cells of a very deficient mouse. Arrows indicate the larger macrophages. 450 ×.

FIG. 6. Pigment-bearing cell with one pole laden with lipid spherules. Arrows indicate surrounding macrophages. 900 ×.

dermal papillae grow down into the dermis as described in the rat by Sullivan and Nicholls.(2) Many sebaceous glands show cellular fragmentation at the periphery. Some mitotic figures can be seen at the periphery of the sebaceous glands of very deficient animals. The cell population of the dermis is increased. Some of the cells appear mesenchymatous, with a dense nucleus and basophilic cytoplasm. Numerous macrophages surround the fundus of sebaceous glands (in normal skin it is almost impossible to recognize macrophages). Mast cells are more numerous than in normal skin. Although some of the mast cells are normal, many possess long filamentous processes. In these cells metachromatic granules are sparsely distributed in the cytoplasm; occasionally they are found only in the cytoplasmic processes. While in normal cells the metachromatic granules are fairly uniform in size, their size is variable in cells where they are scantily disseminated in the cytoplasm. The differences in the state of these cells recall those described by Paff *et al.*(7) in mast cells cultivated *in vitro*. Since Oliver *et al.*(8) have presented evidence that mast cells elaborate heparin, there is a suggestion here of an increase in the amount of heparin released in the dermis of biotin deficient mice. Numerous heterophylic leucocytes and lymphocytes, and a variable number of eosinophiles, also invade the dermis.

The thickened stratum corneum of deficient mice is moderately sudanophilic. The cells of the epidermis possess discrete sudanophilic spherules and rodlets around the nucleus (Fig. 1). The spherules, which contain a sudanophobic center, are usually clustered at one pole of the nucleus. Sudanophilic spherules and perinuclear rodlets are also present in the cells of the bulb and root-sheath of hair follicles and in the ducts of sebaceous glands. In the sebaceous ducts the sudanophilic bodies

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7. Paff, G. H., Bloom, F., and Reilly, C., *J. Exp. Med.*, 1947, v86, 117.

8. Oliver, J., Bloom, F., and Mangieri, C., *J. Exp. Med.*, 1947, v86, 107.

described are more conspicuous. The distribution of the lipid bodies just described is identical with that in the skin of normal mice.

The sebaceous cells of biotin deficient mice, regardless of their position in the gland, contain very large lipid globules (Fig. 3). In nearly all of the glands some peripheral cells show degenerative fragmentation (Fig. 4). The central sebaceous cells seldom show normal sebaceous maturation.

Numerous macrophages, whose cytoplasm is laden with lipid globules or granules, surround the sebaceous glands of deficient animals (Fig. 5). In colored mice, the pigment-bearing cells which surround sebaceous glands also contain variable numbers of lipid droplets (Fig. 6). The numerous mast cells in the dermis contain delicate lipid granules scantily distributed in the cytoplasm.

Although there is a mild reduction in the amount of adipose tissue in the subcutaneous tela, the fatty depots are never depleted as they are in the rat (Sullivan and Nicholls)(2). Furthermore, in some animals there is a fatty infiltration in the dermis.

Sebum is practically absent from the sebaceous glands of very deficient mice. When the pilosebaceous exits have become cystic, the contents of the cysts are meagerly sudanophilic. In deficient animals the sebaceous lipids are usually isotropic. The application of the Liebermann-Burchard test to frozen sections of skin of deficient mice seldom gives a positive reaction in the sebaceous glands.

Discussion. Although the dermatotic symptoms of biotin deficient mice resemble superficially those described by Sullivan and Nicholls(2) in the rat, there are some differences between the rat and the mouse. In deficient rats the skin is covered by a brownish encrustation (Sullivan and Nicholls),(2) but in the mouse it is dry. Keratosis is well developed in the rat but only moderate in the mouse. The pilosebaceous exits are dilated in deficient rats but only rarely in the mouse. The stratum corneum is sudanophilic in deficient rats but it is only mildly so in the mouse. The subcutaneous fatty depots are resorbed in deficient rats, but they remain virtually unchanged in the mouse.

The orifices of the pilosebaceous units in deficient mice are usually plugged by keratinized debris, yet the glands are seldom cystic. This suggests that there is practically no excretion of sebum upon the surface of the skin, and explains the dryness of the skin and the small amount of demonstrable lipid in the stratum corneum of deficient animals.

The sudanophilic inclusions described in the epidermal cells, and in the cells of the pilosebaceous units resemble the lipid part of the Golgi element such as described by Baker(9) and Cain,(10) or the myelin figures of Palade and Claude(11,12) (Fig. 1). Since these and other lipid bodies are more numerous in the cells of the ducts of sebaceous glands of normal and biotin deficient mice, it seems that the early stages in sebaceous accumulation are normal in the skin of biotin deficient mice.

The differences between the sebaceous glands in normal skin and in the skin of biotin deficient animals have been described and are illustrated in Fig. 2, 3, and 4. In biotin deficient skin, the fragmenting sebaceous glands are surrounded by baskets of histiocytes whose cytoplasm is engorged with lipid granules of varying sizes (Fig. 5) whereas in normal skin histiocytes seldom contain lipids. In colored mice, many of the pigment-bearing cells also contain lipid granules and spherules. Lipid inclusions are more abundant in the small pigment-bearing cells than in the larger dermal chromatophores, although some of these also contain demonstrable lipids (Fig. 6). It is not established if these pigment-bearing cells are melanophores which have phagocytized lipids, or if they are dying cells, or if they are macrophages which have engulfed the pigment of fragmented melanophores as well as the lipids released by the breakdown of sebaceous cells. Unlike the situation in man (Masson),(13) lipids are not normally found in the melanoblasts of the mouse. Becker(14)

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9. Baker, J. R., *Quart. J. Micro. Sci.*, 1944, v85, 1.

10. Cain, A. J., *Oxford Sci.*, 1949, v2, 30.

11. Palade, G. E., and Claude, A., *J. Morph.*, 1949, v85, 35.

12. Palade, G. E., and Claude, A., *J. Morph.*, 1949, v85, 71.

13. Masson, P., from The Biology of Melanomas, *Spec. Publ. N. Y. Acad. Sci.*, 1948, v4, 15.

has observed that in human skin the perivascular histiocytes in the dermal papillae phagocytize lipids as well as pigment.

Growth of sebaceous glands and sebaceous accumulation is not impeded by biotin deficiency in mice, but normal storage of lipids in the sebaceous cells and the release of sebum is impaired. Growth of the glands is indicated by the presence of intermediate sebaceous cells and by mitotic activity in the peripheral cells (cf. Montagna and Kenyon(15) for the growth of sebaceous glands in the rabbit). This is in accord with the reports of Bosse and Axelrod(16) who find that wound healing in the skin of biotin deficient rats occurs as rapidly as in normal skin. There is an agreement here also with the conclusions of Wilson and Leduc(3) that in animals deficient in biotin "the capacity of tissue cells for mitosis is not impaired", despite a decline in the mitotic activity in the liver during the development of the deficiency. Abnormal functions of the sebaceous glands, however, is indicated by the absence of brown seborrheal incrustation, the scantiness of sudanophilia in the stratum corneum, the absence of birefringent lipids, the failure of the lipids to give a positive Liebermann-Burchard test, and by the fragmentation of the peripheral sebaceous cells.

Summary and Conclusions. In the mouse the growth potentials of the skin and its appendages are not appreciably affected by biotin deficiency, but normal differentiation and function are impaired. There is a slight increase in mitotic activity in the epidermis and in the sebaceous glands. The pilosebaceous units are blocked by keratinized debris and little or no sebum is released upon the surface of the skin. In the sebaceous cells, the stored lipid droplets are much larger than in those of normal animals. Unlike the sebaceous lipids of normal glands, those of biotin deficient mice are isotropic and negative to the Liebermann-Burchard test. There is abundant cellular fragmentation at the periphery of sebaceous glands and the lipids are apparently phagocytized by histiocytes in the dermis. The melanin-bearing cells around the sebaceous cells, when present, also contain lipid droplets. As a rule, neither histiocytes nor melanin-bearing cells contain demonstrable lipids in the skin of normal mice. There is an increase in the number of mesenchymatous cells, mast cells and leukocytic cells in the dermis of biotin deficient mice.

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14. Becker, S. W., from The Biology of Melanomas, *Spec. Publ. N. Y. Acad. Sci.*, 1948, v4, 82.

15. Montagna, W., and Kenyon, P., *Anat. Rec.*, 1949, v103, 665.

16. Bosse, M. D., and Axelrod, A. E., PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 418.

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Preliminary Observations on the Antiarthritic Effect of 21-Acetoxypregnenolone.* (17601)

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(Introduced by W. E. Ehrich)

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It has been shown by Seifter, Baeder, and Begany(1) that the permeability of the synovial membrane in rabbits is influenced by enzymes and by steroids. Hyaluronidase and desoxycorticosterone (DOCA) were found to markedly increase permeability, while corti-

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* This work was supported by Wyeth, Inc., and Syntex, S. A. (Mexico). Dr. I. V. Sollins of the Chemical Specialties Co., Inc., kindly supplied the 21-acetoxypregnenolone which was used in this study.

1. Seifter, J., Baeder, D. H., and Begany, A. J., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 277.

sone was the most effective in inhibiting permeability through normal and through hyaluronidase treated membranes. Alarm reaction and administration of adrenocorticotrophic hormone were as effective as cortisone. These data were interpreted to mean that normal permeability of the synovial membrane is maintained by a balance between the opposing actions of the two types of adrenal steroids on the ground substance mucopolysaccharides such as hyaluronic acid. According to this view bacterial infection with release of enzymes such as hyaluronidase, or adaptation disease characterized by excess production of DOCA depolymerize the mucopolysaccharides of the ground substance and permit the formation of edema and swelling of the membrane. Cortisone alters the mucopolysaccharides so that they become more resistant to enzymes and to DOCA, their water binding capacity is decreased, and they become freed from edema.

Seifter, Baeder, and Sollins(2) considered the therapeutic effect of cortisone in arthritis to consist of at least 3 phases: (1) immediate anti-enzyme or anti-DOCA effect on the synovial membrane (end organ) resulting in alleviation of edema and swelling; (2) healing action on synoviae possibly by general effects on carbohydrate metabolism; and (3) general metabolic effect on the rheumatoid state. They employed the rabbit synovial membrane technic to screen more than 20 steroids[†] and to compare their efficiency with cortisone on the end organ. The barrier effect of cortisone was rated as 100%. On this basis, the barrier of the normal membrane had a 4.5% cortisone rating. All but 2 of the steroids screened decreased permeability of the synovial membrane. Pregnenolone, testosterone, combinations of these, and most of the other steroids had less than 40% cortisone effect. Two steroids were found to be at least as active as cortisone. One of these, 21-acetoxypregnenolone, had activity 130% that of cortisone. It also antagonized the effect of DOCA and hyaluronidase[‡] on the synovial membrane. The anti-DOCA effect was five times as great as the anti-hyaluronidase effect. Comparison of the formula of this compound with that of DOCA indicated resemblance of structure sufficient to warrant consideration of its action as a competitive inhibitor of DOCA.

We decided to employ 21-acetoxypregnenolone as an investigative tool to determine whether the simple end organ effect would be sufficient to alleviate rheumatoid arthritis or whether the other actions of cortisone contribute an important role in the treatment of this disease. In this paper we are reporting the preliminary observations which confirm the findings in the rabbit studies. Evaluation of 21-acetoxypregnenolone as a treatment is being carried out on a separate group of hospitalized patients on whom the customary laboratory investigations of the rheumatoid state and necessary metabolic studies are being carried out.

Material and method. Seven patients were selected for this preliminary study according to the following criteria: (1) The patient must have rheumatoid arthritis of a moderate or marked degree, (2) the rheumatic process must be demonstrably active, and (3) the patient must be ambulatory and engaged in his usual activity. One patient, case No. 7, was hospitalized and placed at bed rest. Ambulant cases were selected in an attempt to minimize the known beneficial effects of bed rest. All patients had previously received routine arthritic management. With the exception of case No. 6, each patient was given 100 mg cholesterol daily by intramuscular injection for 3 days prior to the administration of 21-acetoxypregnenolone. 21-Acetoxypregnenolone was then administered intramuscularly, 100 mg the first day, 200 mg on the second day, and 300 mg daily thereafter for a total period of 14 days. At the end of this period the dosage was decreased to 200 mg, and the interval was lengthened to 2 days. Each patient was examined daily by one of us (D.R.F.) and at 3 day intervals by all of

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2. Seifter, J., Baeder, D. H., and Sollins, I. V., to be published.

[†] These compounds were prepared by Doctors John Pataki, Stephen Kaufmann, and George Rosenkranz of the Research Laboratories, Syntex, S.A.

[‡] Hydase, Wyeth.

us. Improvement in overall function, swelling, pain, local inflammation, and joint tenderness was measured in accordance with the Criteria for Gauging Rheumatic Improvement as agreed upon by the New York Rheumatism Association: grade 0 = no improvement; grade + = slight; grade ++ = moderate; grade +++ = marked; grade ++++ = very marked.

Case No. 1, M. H. Age: 39. Occupation: typist. Diagnosis: rheumatoid arthritis. Duration of disease: 2½ years. Joints chiefly involved: both shoulders, and interphalangeal joints of both hands. No change in subjective or objective findings was noted during 3 days of cholesterol administration. Significant improvement first noted on 4th day of 21-acetoxypregnenolone administration. Grade +++ improvement in pain, tenderness, and function noted by all observers on 5th day. Improvement continued over 2 weeks period and patient stated that typing had greatly improved, fatigue had lessened, and sense of well being had improved; improvement was rated as grade ++++. Slight return of swelling and tenderness accompanied reduction of dosage and lengthening of interval. Weight gain of 2 lbs in 2 weeks was noted. No evidence of fluid retention.

Case No. 2, H. B. Age: 34. Occupation: housewife. Diagnosis: rheumatoid arthritis. Duration of disease: 16 years. Joints chiefly involved: both knees, left elbow, and right wrist. Patient reported an aggravation of symptoms during three days of cholesterol therapy. Significant improvement first noted on third day of 21-acetoxypregnenolone administration. Improvement in euphoria, function, pain, tenderness, and swelling was rated as grade +++ on 5th day. Improvement over 2 weeks period was rated as grade ++++ and patient was able to climb stairs for first time in 2 years. Reduction in dosage and lengthening of interval did not cause regression in symptoms. Patient gained 1½ lbs in two weeks period and showed no evidence of fluid retention.

Case No. 3, N.N. Age: 56. Occupation: housewife. Diagnosis: rheumatoid arthritis. Duration of disease: 5 years. Joints chiefly involved: left wrist and left knee. Patient reported aggravation of symptoms during 3 days administration of cholesterol. Significant improvement first noted on 3rd day of 21-acetoxypregnenolone administration. Evaluation on the 5th day revealed remarkable reduction in swelling and grade +++ improvement in local inflammation, tenderness, pain, function, and general mood. By the 8th day patient reported that she had no arthritic symptoms. Evaluation of improvement at end of 2 weeks period revealed grade ++++ improvement in all phases. Reduction in dosage and lengthening of interval was attended by only a slight regression in previously noted improvement. Patient had a total weight gain of 1 lb in two weeks and showed no evidence of fluid retention.

Case No. 4, A. S. Age: 43. Occupation: housewife. Diagnosis: rheumatoid arthritis. Duration of disease: 8 years. Joints chiefly involved: both shoulders, right wrist, right elbow, and metacarpophalangeal joints. This patient was in a severe relapse at the start of treatment and was becoming rapidly worse. Three days of cholesterol therapy seemed to aggravate symptoms. First significant improvement was noted on the 8th day of treatment with 21-acetoxypregnenolone. Evaluation at end of 2 weeks period was rated as grade +++ improvement in general function and in all joints. Reduction in dose and lengthening of interval resulted in a moderate relapse in previously noted improvement. Patient gained no weight during two weeks interval and there was no evidence of fluid retention.

Case No. 5, M. W. Age: 65. Occupation: housewife. Diagnosis: rheumatoid arthritis. Duration of disease: 7 years. Joints chiefly involved: knees, right shoulder, wrists, and metacarpophalangeal joints. Increased fatigue and no improvement in joint symptoms noted during 3 days cholesterol therapy. First significant improvement occurred on 3rd day of treatment with 21-acetoxypregnenolone, with full motion of right shoulder unattended by pain. Improvement in general function, mood, and all joints was rated as grade ++++ on the 5th day of therapy. Improvement continued over two weeks period despite great increase in daily activity. Patient gained 2½ lbs over two weeks period and there was no evidence of fluid retention. Patient noted a slight decrease in functional ability of right hand on reduction of dosage and increase in interval.

Case No. 6, M.C. Age: 46. Occupation: dietitian. Diagnosis: rheumatoid arthritis. Duration of disease: 38 years. All joints involved. Many joints "burned-out" and ankylosed. Clinical activity most pronounced in right knee. Marked soft tissue swelling in hands and knees with grade II dependent edema in ankles. Patient not given cholesterol but started immediately on 21-acetoxypregnenolone, 300 mg daily. Reduction in swelling noted 48 hours after onset of therapy. Grade +++ reduction in pain and swelling of right knee noted after 3 days of therapy.

Case No. 7, S. K. Age: 19. Occupation: student. Diagnosis: rheumatoid arthritis. Duration of disease: 3½ years. Joints chiefly involved: knees, metacarpophalangeal joints and interphalangeal joints. This patient was hospitalized for metabolic study, which will be included in a later report. No improvement during 3 days of cholesterol therapy. Significant improvement first noted on 3rd day of administration of 21-acetoxypregnenolone. Evaluation on the 5th day of therapy revealed grade ++++ improvement in swelling, function, and tenderness. Patient noted only minimal stiffness upon arising in morning.

Summary and conclusions. 1. 21-Acetoxypregnenolone was selected for a limited study in 7 ambulatory patients with rheumatoid arthritis because in the rabbit synovial membrane screen it was at least as good as cortisone in decreasing permeability and it showed powerful anti-DOCA action on synovial membrane.

2. The effect on the end organ seen in the screen in rabbits manifested itself in each of the seven patients by reduction in swelling, lessening of the pain, and increased motion in the joints.

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Inotropic Action of Epinephrine, Nor-epinephrine, and N-Isopropyl-Norepinephrine on Heart Muscle.*† (17602)

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In a recent report, Goldenberg *et al.*(1) stated that "nor-epinephrine hypertension is due to an increase of total peripheral resistance, with no significant change, or even a drop in cardiac output whereas epinephrine hypertension is the result of a significant increase of cardiac output." It is not clear which of the factors that determine cardiac output are responsible for the difference of response to epinephrine and nor-epinephrine. One factor which can be evaluated is the effect of the two substances on the contractile force of myocardium. Krop(2) has shown that epinephrine exerts a marked inotropic action on heart muscle, but similar studies have not been reported with nor-epinephrine. Therefore, a comparison was made of the effects of U.S.P. epinephrine, d-l nor-epinephrine (Arterenol) and N-isopropyl-nor-epinephrine (Isuprel) on the contractile force of heart muscle, in an attempt to determine whether the differences in cardiac output reported by Goldenberg *et al.* are due to a lack of inotropic effect by nor-epinephrine.

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† The author wishes to thank Dr. M. L. Tainter of the Sterling-Winthrop Research Institute who supplied the d-l nor-epinephrine and the N-isopropyl-nor-epinephrine.

1. Goldenberg, M., Pines, K. L., Baldwin, E. deF., Greene, D. G., and Roh, E. C., *Am. J. Med.*, 1948, v5, 792.

2. Krop, S., *J. Pharm. and Exp. Therap.*, 1944, v82, 48.

TABLE I.
Increase in Contractile Force of Heart Muscle Produced by 3 Sympathomimetic Amines.

Muscle	Epinephrine		d-l Nor-Epinephrine		N-Isopropyl-Norepinephrine	
	$1:10^7$ %	$1:10^6$ %	$1:10^7$ %	$1:10^6$ %	$1:10^7$ %	$1:10^6$ %
1	12	45	23	63	44	60
2	13	20	9	14	9	17
3	57	86	139	209	89	176
4	40	70	67	111	71	83
5	79	126	94	169	83	117
Avg	40	69	66	113	59	91

Method. The papillary muscle preparation of Cattell and Gold(3) was used, since it permits the measurement of contractile force without the complications attending studies on intact animals or isolated hearts. The chorda tendina of the isolated muscle was attached to a strain gauge whose output was recorded by a Grass electroencephalograph. The muscle was immersed in oxygenated Locke's solution, and stimulated electrically at a rate of 30 per minute. A series of 5 muscles was studied. After an equilibration period of at least 30 minutes, the contractile force was measured, and a drug added to produce a total concentration of 1:10,000,000. After 2 minutes, the contractile force was measured again, and the concentration of the drug was then raised to 1:1,000,000, and further measurements made, before the onset of a spontaneous rhythm. The drug was then removed, the chamber rinsed, and fresh Locke's solution added. After another equilibration period of at least 15 minutes, a new control reading was taken, and the procedure repeated with another drug. The sequence in which the drugs were tested on the 5 muscles was varied.

Results. The results were expressed as percentage of increase of the control force of contraction (Table I). All of the drugs studied produced an increase in the force of contraction of each muscle. There was a considerable variation in the degree of the increase, so that accurate quantitative relationships between the drugs cannot be derived from this study. Nevertheless it is clear that both d-l nor-epinephrine and N-isopropyl-nor-epinephrine exert an inotropic action on heart muscle which is at least as great as that produced by U.S.P. epinephrine.

Discussion. Since many factors other than contractile force determine cardiac output, these results cannot be interpreted as evidence against the conclusions of Goldenberg *et al.*(1) However, they indicate that the differences in cardiac output which these authors reported are probably not due to a lack of inotropic effect by d-l nor-epinephrine.

Summary and conclusions. d-l Nor-epinephrine (Arterenol) and the N-isopropyl-norepinephrine (Isuprel) exert a powerful inotropic action on mammalian heart muscle.

Received December 19, 1949. P.S.E.B.M., 1950, v73.

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3. Cattell, McK., and Gold, H., *J. Pharm. and Exp. Therap.*, 1938, v62, 116.

Protein and Arginine Levels in Chick Diets. (17603)

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A further investigation was conducted on the arginine requirement of the growing chick, particularly as affected by protein level. Diets used and procedures followed were very similar to those recently described(1) and need not be given in detail. In the present case, all diets contained 5% of dehydrated alfalfa meal and sufficient of a good grade of commercial casein to furnish the desired protein levels as determined by analysis. In addition to all known vitamins and minerals, necessary quantities of glycine, methionine, and animal protein factors* were added. The arginine content of the casein and alfalfa protein was taken as 4.0% of the crude protein (%N × 6.25).

The results are given in Table I. It is evident that in this case, as well as in the previous methionine(1) and lysine(2) studies, the indispensable amino acid requirement (chick) increases with the protein level in the diet. As a per cent of the protein, the arginine requirement is not more than 6% at each protein level. At each level of protein, the next amount of arginine below 6% of the protein appears less than optimal. As closely as the data can be interpreted, the arginine requirement is a practically constant proportion of the protein.

In agreement with the former report,(1) the highest protein level supported the highest growth rate, although the chicks were somewhat older in the present work. In a previous review,(3) the arginine level for optimal growth was indicated as 1.2% of the diet, at approximately 20% of protein in the diet. This indication is supported by the present data. The general discussion of results in a former publication(1) applies to the present report, as well.

Summary. The arginine requirement of the chick was estimated in relation to the protein level in the diet, from 15 to 30%. The requirement was found to be approximately 6% of the total protein in the diet, at each level of protein.

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TABLE I.
Relation of Protein and Arginine Levels to the Rate of Gain of Chicks.

Protein in diet, %	Arginine added to diet, %	Total arginine in diet, %	Arginine in protein, %	Daily rate of gain, %
15	0.0	.6	4.00	5.38
15	.1	.7	4.67	5.48
15	.2	.8	5.33	5.68
15	.3	.9	6.00	5.86
15	.4	1.0	6.67	5.75
20	0.0	.8	4	5.97
20	.2	1.0	5	6.14
20	.4	1.2	6	6.39
20	.6	1.4	7	6.40
20	.8	1.6	8	6.41
30	0.0	1.2	4	6.38
30	.3	1.5	5	6.67
30	.6	1.8	6	6.95
30	.9	2.1	7	6.90
30	1.2	2.4	8	6.96

Conducted with selected Barred Rock-New Hampshire cross chicks about 4 weeks old and kept on experimental diets for 10 days. 8 chicks per group.

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* Experimental preparation N 199 B, Lederle Laboratories. Kindly provided by Dr. T. H. Jukes.

2. Grau, C. R., *J. Nutrition*, 1948, v36, 99.
3. Almquist, H. J., *J. Nutrition*, 1947, v34, 543.

Role of Mucopolysaccharides in Pathogenesis of Experimental Exophthalmos.* (17604)

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In the course of investigations of hormonal effects on connective tissue in these laboratories, our attention was drawn to the problem of localized myxedema. As a result of our clinical impression that this lesion was often associated with progressive exophthalmos, the studies to be reported here were initiated. Recently, Curtis(1) has also pointed out that the two conditions occur frequently in the same patient, and have a very similar clinical course.

The primary pathological change in localized myxedema is an infiltration of the dermis by a mucinous intercellular and interfibrillar ground substance,(2,3) Watson and Pearce,(4) by direct chemical analysis of skin from patients with pretibial myxedema, demonstrated a marked increase in hyaluronic acid, chondroitin sulfuric acid, and water in the affected areas.

Experimental exophthalmos has been produced in guinea pigs by several investigators both by thyroidectomy and with extracts of the adenohypophysis containing the thyroid stimulating hormone (TSH)(5). Smelser(6,7) reported that the orbital tissues of such animals were edematous and contained a stainable amorphous material in the connective tissue. Subsequently, he demonstrated that the increase in weight of the orbital contents was due to an increase in water content, and suggested that edema of these tissues is responsible for the forward proptosis of the eye.

On the basis of these observations, it was decided to study the amounts of mucinous substances in the orbital tissues in experimental exophthalmos. In addition to histochemical studies, water and hexosamine contents were determined. Since mucopolysaccharides contain fixed quantities of hexosamines, the determination of these substances may be used as an index of the amount of mucopolysaccharide present.

Methods. Four sets of young guinea pigs (250 g) were studied: A) Intact controls, B) Intact animals treated with thyroid stimulating fraction from the adenohypophysis (TSH), C) Thyroidectomized controls, and D) Thyroidectomized animals treated with TSH. The animals were fed a stock diet of Purina Dog Chow with additional fresh greens, excluding cabbage. Those receiving TSH‡ were injected intraperitoneally with 25-30 Junkmann-Schoeller units per day. The degree of exophthalmos was estimated grossly. The animals were etherized and sacrificed by exsanguination at intervals up to 3 weeks after the start of the experiment. The entire orbital contents were removed and the eyes were carefully dissected free and discarded. The lacrimal and Harderian glands were separated from the remaining tissues, which consisted of the extraocular muscles, adipose tissue and loose connective tissue. The contents of one orbit were then fixed in formalin and absolute alcohol, while the other was saved for meas-

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† Charles Klingenstein Fellow.

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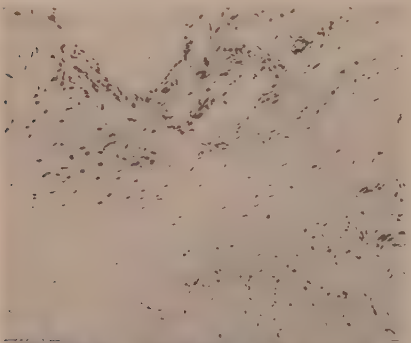
‡ The TSH used was Thyrotropic Hormone, Parke Davis and Co., R No. 099802, 6-8 Junkmann-Schoeller units per mg, obtained through the courtesy of Dr. D. A. McGinty.

urement of water and hexosamine content. The fixed material was sectioned at five micra and stained with hematoxylin and eosin, and toluidine blue. Sections of the material were digested in a solution of bull testis hyaluronidase[§] containing 50 turbidity reducing units per ml. To estimate water content, the tissues were placed in tared watch glasses and weighed on an analytical balance immediately after removal from the orbit. They were then dried in an oven at 100°C for 12 hours and kept dry in a vacuum desiccator over $CaCl_2$ until reweighing. The hexosamine content was determined by an adaptation to whole tissues of West's(8) modification for serum of the method originally described by Elsom and Morgan.(9) The dried tissues were transferred to 10 ml volumetric flasks and hydrolyzed for 6 hours in a boiling water bath after the addition of 3 ml of 2N HCl. After cooling, the flasks were filled to the 10 ml mark with distilled water, and the contents filtered through a No. 5 Whatman filter. 0.5 ml aliquots of the filtrate were then carried through as described by West. The hexosamine values were expressed as mg per 100 mg of tissue (dry weight), using a glucosamine standard.



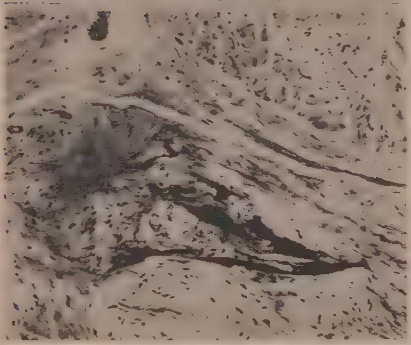


FIG. 1.
Section of extraocular muscle and connective tissue from a control animal, showing the faint metachromasia. Toluidine blue stain. × 125.

FIG. 2.
Section of similar area from thyroidectomized guinea pig treated with TSH. Note heavy deposition of intensely metachromatic material. Toluidine blue stain. × 125.

Results. None of the animals in the control group developed exophthalmos. Histologic examination revealed extremely small amounts of metachromatic material scattered throughout the connective tissue and in the capsule of the Harderian gland (Fig. 1).

Exophthalmos appeared in the intact animals receiving TSH (Group B) within 2 to 3 days after the onset of treatment, began to diminish within 10 to 12 days, and disappeared almost entirely after 3 weeks. The thyroidectomized control animals (Group C) developed moderate exophthalmos. All the thyroidectomized animals receiving TSH (Group D) developed marked exophthalmos. The orbital tissues of this group were distinctly mucoid and viscous on gross examination.

The histologic findings paralleled the macroscopic observations. All animals with exophthalmos showed large deposits of intensely metachromatic ground substance in the connective tissue, particularly in the fat tissue, the fasciae of the extra-ocular muscles, and the capsules of the Harderian and lacrimal glands. (Fig. 2 and 3). In Group D, where the extent of the changes was greatest, there were actual lakes of this material and metachromasia in the connective tissue between the muscle fibers and in the septa of the glands. Digestion with hyaluronidase resulted in almost complete removal of the metachromatic deposits. (Fig. 4) Lymphoid nodules in the

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[§] Bull testis hyaluronidase supplied by Dr. E. Seifter, of the Wyeth Research Laboratories, Philadelphia, Pa.

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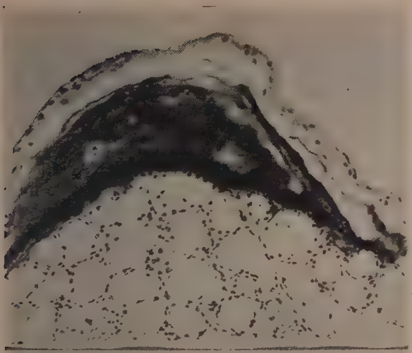

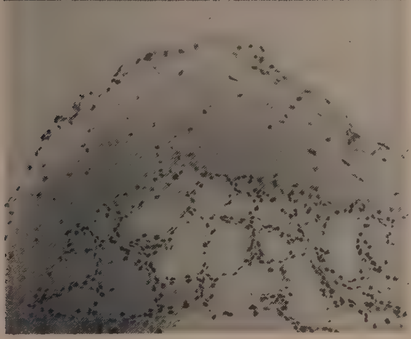

Fig. 3.
Section of Harderian gland from thyroidectomized guinea pig treated with TSH, with large amounts of mucopolysaccharides in the capsule. Toluidine blue stain. × 125.

Fig. 4.
Adjoining section from same animal after hyaluronidase digestion. Toluidine blue stain. × 125.

subconjunctival area, seen in the controls, were considerably enlarged in the other groups, but no lymphocytic infiltration of the connective tissue was observed. One section revealed a small focus of muscle degeneration.

Water and hexosamine determinations were carried out on all three components into which the retro-ocular tissues were divided, the lacrimal gland, Harderian gland, and the muscle-fat fraction. The changes were most striking and clear-cut in the latter fraction, because of the presence of a greater proportion of connective tissue. Accordingly, only the data obtained from this tissue will be presented now. The water contents of these tissues correlated well with the degree of metachromasia and the degree of exophthalmos. The most striking and significant increase occurred in the thyroidectomized group given TSH (Group D). The intact group receiving TSH (Group B) exhibited a moderate increase at first, followed by a fall to control levels. (Table I)

The hexosamine concentrations also varied directly with the degree of exophthalmos, metachromasia, and water content. (Table I) It, too, was most strikingly elevated in Group D and less so in the other groups. Group B exhibited an initial rise, but the subsequent fall was less striking than the secondary decreases in the other factors studied. The increase in water and hexosamine content in the treated animals cannot be ascribed to proliferation of the orbital tissues, since analysis of the total dry weights of the orbital contents in the four groups reveals an actual decrease in the weights of the retro-ocular tissues in Groups C and D in relation to the control group. (Table I) This indicates that in this experiment exophthalmos is independent of the growth of the orbital tissues.

Discussion. These results indicate that the pathologic changes observed in exophthalmos are similar to those of localized myxedema and consist essentially of the accumulation of interfibrillar ground substance in the orbital connective tissue. The response to hyaluronidase digestion indicates that a large proportion of the mucopolysaccharide moiety of this ground substance is hyaluronic acid. It is accepted that the mucopolysaccharides are extremely hydrophilic (10) and, when present, play a major rôle in the maintenance of tissue water content. The large amounts of these substances in the orbital tissues in experimental exophthalmos, therefore, must contribute significantly to the increase in water content reported by Smelser,(7) and confirmed by us. It would appear, therefore, that the increase in volume of the orbital contents in experimental exophthalmos is the result of both an accumulation of mucopolysaccharides and the resultant rise in water content. In the rigid confines of the orbit, the increased volume of the retro-orbital tissues is in turn

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10. Ropes, M. W., Roberts, W. vB., Rossmeisl, E. C., Peabody, R. B., and Bauer, W., *Acta med. Scandinav.*, 1947, Suppl. 196, 700.

TABLE I.
Summary of Analyses of Orbital Contents of Guinea Pigs.

	Group	No. animals	Water content, %	Hexosamine content, mg/100 mg tissue (dry wt)	Total dry wt orbital content
A	Intact controls	12	80.6 ± .4*	.70 ± .03	145.5 ± 6.4
B	Intact + TSH (5 days)	4	82.7 ± .8	.97 ± .05	156.9 ± 7.3
B	Intact and TSH (14 and 21 days)	8	80.5 ± 4	.90 ± 09	
C	Thyroidectomized	5	83.1 ± .2	.92 ± .07	122.9 ± 15.5
D	Thyroidectomized + TSH	8	86.9 ± .5	1.27 ± .11	109.7 ± 8.9

$$* \text{S.E.} = \sqrt{\frac{\Sigma\Delta^2}{n(n-1)}}$$

responsible for the extrusion of the ocular globe.

Summary. The development of experimental exophthalmos in the guinea pig is dependent on the accumulation of large quantities of intercellular ground substance and water in the connective tissue of the orbital contents. This is evidenced by the presence of considerable amounts of metachromatic material, of which hyaluronic acid is an important component, and by an increase in the hexosamine and water content of these tissues.

This is similar to the changes observed in localized myxedema in man, and suggests a common mechanism for the two conditions.

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Inhibition of Aerobic Respiration of Rat Brain by Desoxycorticosterone *in vitro*.* (17605)

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Selye's observation that large amounts of steroids induce anesthesia,(1) together with the relationships thought to exist between anesthetics and the carbohydrate metabolism of brain,(2) have provided a basis for studies concerning the effect of steroids on rat brain cell respiration. It has been found that these agents are capable of inhibiting the aerobic respiration of rat brain, presumably at the dehydrogenase level.(3) Our previous studies indicate that this phenomenon also operates in the intact organism since (a) deprivation of gonadal steroids by castration leads to an increased rate of glucose oxidation by rat brain,(4) (b) tissues from castrated male rats differ from normal tissues in their response to steroids added *in vitro*,(5) and (c) a parallelism can be shown between the *in vitro* inhibiting potency and *in vivo* anesthetic activity of the steroids.(3)

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* Aided by grants from the National Institutes of Health, Bethesda, Md., and from Ciba Pharmaceutical Products, Inc., Summit, N.J.

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2. Michaelis, M., and Quastel, J. H., *Biochem. J.*, 1941, v35, 518.
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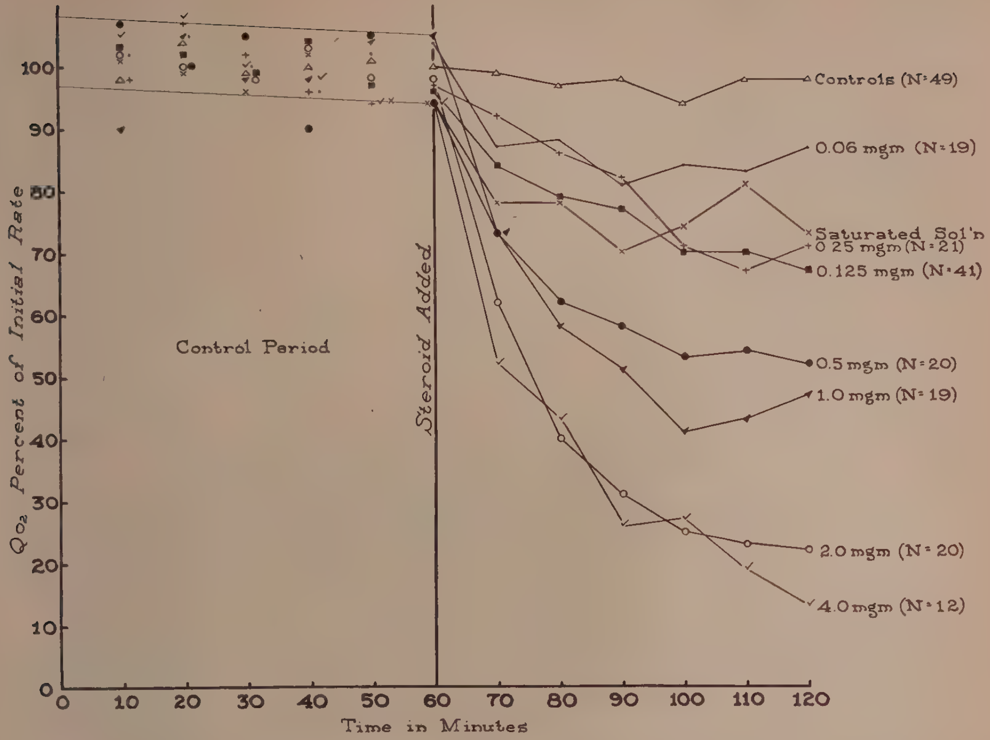

FIG. 1.
Effect of DC upon respiration of rat brain suspensions *in vitro*. (N represents number of vessels).

We have been concerned, however, by the relatively large amounts of steroid used to produce this effect upon aerobic respiration. It was our original assumption that the low aqueous solubility of the compounds would cause any excess to act simply as a reservoir, and that the solution in the flask would be saturated or supersaturated as long as any excess were present. This assumption led to the use of large amounts of suspended steroid as well as the selection of a tissue with a high lipid content dispersed as a cellular suspension to increase surface area. To study this problem further, we have compared the effect of varying concentrations of a steroid upon the oxygen consumption of a standard rat brain cell suspension. Inasmuch as desoxycorticosterone (DC) produces more inhibition than other steroids we have studied, it was selected as a steroid suitable for comparison of concentration-action relationships. Comparisons were also made using similar amounts of a water-soluble conjugate, desoxycorticosterone glucoside (DCG).†

Method. Cerebral hemispheres obtained from Slonaker-Wistar rats were made into a 10% suspension in modified glucose Ringer phosphate buffer using the Potter all-glass homogenizer. Oxygen uptake was determined at 10-minute intervals using conventional manometric technic. DC, previously ground to a fine powder and suspended in 0.2 ml of distilled water, was added from a side arm after a 60-minute control period. Readings were then made for an additional 60 min. Each flask contained 1 ml (100 mg) of brain suspension in a total final volume of 2 ml. In other experiments, aqueous solutions of DCG were similarly added.

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† The steroids used in this study were generously furnished by Doctor E. Oppenheimer, Ciba Pharmaceutical Products, Inc., Summit, N.J.

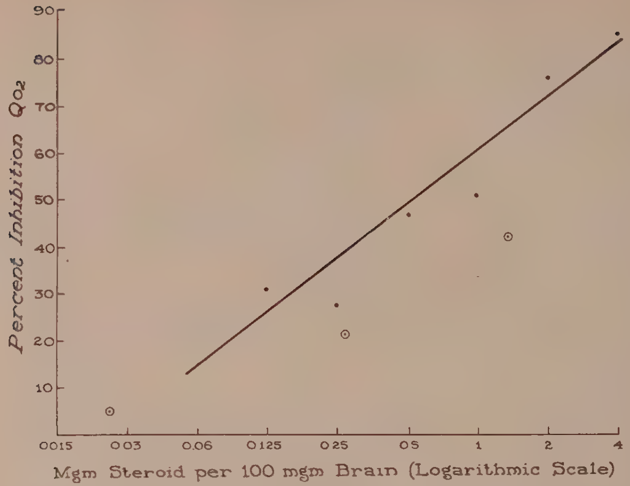

FIG. 2.

Relation between amount of desoxycorticosterone and per cent inhibition of respiration. (Solid points represent DC, circled points, DCG.)

Experimental Data for DC are plotted in Fig. 1. It will be observed that increasing amounts of steroid produce increasing degrees of inhibition. Fig. 1 also demonstrates that increasing amounts of steroid take increasingly longer periods of time to attain a maximum effect. Maximal effect, once obtained, is constant with no increase or release of this inhibition of respiration even when the experimental period is extended to 2 hours. The concentration-effect relationship seen in Fig. 1 is reasonably linear when the logarithm of the amount of steroid is plotted against the per cent inhibition (Fig. 2). Analysis of variance for the data shows no evidence of deviation from linearity. The per cent inhibition for each amount of steroid was calculated by comparing the QO_2 one hour after addition of the steroid with the average QO_2 for the 60-minute control period. This figure was corrected by subtracting the 2% drop in QO_2 observed in control vessels over the same period of time. DCG likewise shows increasing effect with larger amounts in the range studied. These points are shown in Fig. 2 as the amount of DC added (calculated from the relative molecular weights of the free steroid and the glucoside conjugate). The glucoside appears to be somewhat less potent, but this difference is difficult to assess because of a drop in QO_2 in the control flasks of this experiment.

Examination of Fig. 2 demonstrates that 0.2 ml of a saturated solution of DC produces an effect biologically comparable to that produced by 0.24 mg of the steroid (indicating a solubility of 3.6×10^{-3} M). The solubility of DC has been reported as 3.6×10^{-4} M.(6) The discrepancy between these solubilities is probably accounted for by the difference in the temperatures at which they were obtained, the larger figure being determined at 37.2°C and the smaller at 18°C.

Discussion. These data indicate that our previous hypothesis of the large amounts of steroid acting simply as a reservoir is no longer tenable. If that supposition had been correct, there should have been little difference in effect from the varying amounts used. In addition, the water-soluble conjugate produced a degree of inhibition similar to that found with the equimolar amount of unesterified steroid and in a shorter period of time. Increasing amounts of the conjugate likewise produced increasing effects.

It also appeared that the larger amounts of steroid require a longer period of time to

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6. Miescher, K., Fischer, W. H., and Meystre, C., *Helv. Chim. Acta*, 1942, v25, 40.

affect the tissue, but eventually do so, leaving no reservoir of steroid in the liquid phase. To test the validity of this conclusion, the preparations resulting from 60-minute incubation of the brain preparation with 1, 2, and 4 mg of DC at 37.2°C were filtered and the filtrates examined for steroid by Zimmerman reaction and for effect on respiration of other brain suspensions. No steroid could be detected in the filtrate by either method. The amount of DC necessary to produce minimal significant inhition of aerobic respiration *in vitro* was 60 μg, a greater concentration than that which has been shown to affect other systems. For example, Bartlett and Mackay(7) have found that 30 μg of DC reduce the amount of glycogen formed when skeletal muscle is incubated with glucose.

Summary. (1) The concentration-action relationships of DC and DCG upon aerobic respiration of rat brain cell suspensions indicate:

(a) Increasing amounts of DC and DCG produce increasing amounts of inhibition This relationship is reasonably linear plotting the logarithm of the amount of DC against per cent of inhibition produced.

(b) With increasing amounts of DC a longer period of time is required to attain maximal inhibition while a comparable effect is more rapidly attained with equimolar amounts of the water-soluble conjugate, DCG. The effect, once attained, remains constant without further increase or release of the inhibition.

(c) By this bio-assay the water-solubility of DC at 37.2°C is approximately 3.6×10^{-3} Molar.

(2) These data suggest that, under the conditions used, the relatively large amounts of steroid necessary to produce inhibition of aerobic respiration *in vitro* entirely penetrate or are adsorbed by the affected system. These amounts exceed the concentrations of steroid which will affect other systems *in vitro* or that occur in living organisms. This does not necessarily exclude the possibility that steroidal inhibition of aerobic respiration may be of significance in the intact organism.

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Specific Localization of Anti-Rat-Lung Serum in the Lung.* (17606)

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Up to the present time, the only direct evidence that antibodies prepared against an animal's tissue actually localize in the homologous tissue has been the demonstration, with the use of radioactive tracers, that antibodies prepared in rabbits against rat kidney or mouse kidney do localize in the kidneys of rats or mice.(1-5) In order to observe whether tissues other than kidney can concentrate antibodies from tissue-homologous antisera, or whether kidney is unique in this property, we have investigated antisera against several other rat tissues.

We now have evidence, which we are reporting here, that antibodies prepared against rat lung localize in the lung. Experiments were also carried out with antisera prepared against rat brain, liver, and heart and with a control non-tissue antiserum, namely, antiovalbumin. No specific localization was observed with these anti-tissue sera as compared to the antiovalbumin control. However, we have reason to believe, as discussed presently, that antisera prepared differently may yield positive localization in some of these tissues, also.

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* Paper VII in the series, The Zone of Localization of Antibodies. For Paper VI, see reference 1. This research was aided by grants from the Office of Naval Research and the James Foundation of New York, Inc.

† Senior Fellow, in Cancer Research, American Cancer Society Fellowship recommended by the Committee on Growth of the National Research Council.

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3. Pressman, D., *Cancer*, 1949, v3, 697.
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The anti-rat-tissue sera were prepared in rabbits by the injection of antigens obtained by homogenizing the perfused tissue(2) of rats of the S. C. Wistar strain supplied by Dr. Deuel of the University of Southern California. The anti-rat-kidney serum and the antiovalbumin serum were the preparations described previously.(2) For the production of anti-rat-lung, -brain, and -heart sera, two rabbits, in the case of each tissue, were injected intraäbdominally twice a week on consecutive days for 3 weeks with 5% suspensions of brain or lung or a 2½% suspension of heart. The animals were bled from the ear one week after the last injection. A subsequent bleeding was obtained after a second course of 2 weeks of 2 injections per week of 2 ml of a 15% suspension of the tissue. The sera of the 2 bleedings for the 2 rabbits were pooled since they all showed similar titers in precipitation reactions. The anti-liver serum was prepared by injecting 2 rabbits intraäbdominally twice a week on consecutive days for 2 weeks with a 30% suspension of liver tissue and bleeding the animals one week after the last injection. The globulin fractions of these sera were prepared by the alcohol fractionation method described previously.(3) All of the sera and the globulin fractions thus prepared showed precipitation when they were titered with the homologous antigen. There was appreciable cross reaction with other anti-tissue sera, but no precipitate was obtained with these antigens and normal serum. The antigens for this test were prepared by centrifuging a 10% suspension of the perfused tissue for 30 minutes at 1000 RCF and using the somewhat cloudy supernate. Both S. C. Wistar and Sherman albino rats were used to supply the test antigen preparations.

The globulin fractions of the antisera were iodinated with iodine containing tracer amounts of the radioactive I^{131} as described previously.[‡](4)

Each of the radioiodinated globuin fractions of the antisera was injected intravenously into several Sherman albino rats of about 150 g weight. About 0.7 mg of protein and 10 μc (1.4×10^6 counts per min.) of radio-activity in 0.5 ml of solution was used for each injection. At intervals of 3, 6, 10, 13, 18, and 25 days after injection, one animal in each group was sacrificed, perfused, and the tissues analyzed for radioactivity as described elsewhere.(3)

The results are shown in Table I where the concentrations of radioactivity (counts per minute per gram) of blood or of wet perfused tissue, *i.e.*, lung, kidney, liver, brain, heart, and spleen on the various days after injection are tabulated for the different antisera used.

In the case of the lung, a much higher concentration of radioactivity localized from the anti-lung serum than from any of the other sera used. Indeed, none of the other anti-tissue sera were significantly more active in this respect than the antiovalbumin control. In the case of the kidney, there was high concentration of radioactivity localized from the anti-kidney serum as was described previously(2) and an essentially equivalent high concentration of radioactivity from the anti-lung serum. The kidney-localizing antibody in the anti-lung serum appeared to be quite similar, with respect to extent and duration of localization, to the kidney-localizing antibody in the anti-kidney serum. The extents of localization of the anti-lung serum in the kidney and in the lung were essentially the same initially, but the duration was much less in the lung than in the kidney so that the relative concentration in the kidney became greater with time. The anti-brain and anti-heart sera seemed to show very slightly greater localization in kidney than did the anti-liver serum and the antiovalbumin serum control. This greater localization may have been real but it was still much less than that observed with anti-kidney and anti-lung sera.

[‡] Carrier-free radioiodine was obtained from the U. S. Atomic Energy Commission, Oak Ridge Operations, Isotopes Division, Oak Ridge, Tenn.

TABLE I.

Localization of Radioactivity in Various Tissues from Radioiodinated Globulin Fractions of Various Anti-tissue Sera.

Sherman albino rats were injected intravenously with about 0.7 mg protein and 10 μc (1.4×10^6 counts per minute). The animals were perfused on the days indicated and the tissues analyzed.

Tissue	Antiserum used	Radioactivity in tissues (Counts per g per min.)*					
		3rd day	6th day	10th day	13th day	18th day	25th day
Lung	Anti-l†	8650	2390	1000	320	230	(44)
	,, k	2400	1280	320			(21)
	,, li	2230	270	(85)	(40)	(13)	(0)
	,, b		380	230			
	,, h	1350	630	310			
	,, o	1880	770	(60)	(20)	(13)	(8)
Kidney	Anti-l	9380	5900	4180	2670	1480	770
	k	11200	6700	7050			920
	,, li	1980	550	380	350	230	(30)
	,, b	2760	1760	1020			
	,, h	1150	2140	1200			
	,, o	680	390	650	870	290	(42)
Liver	Anti-l	3340	1540	580	230	300	100
	,, k	2170	970	640			50
	,, li	3340	570	130	64	40	(0)
	,, b	3400	820	270			
	,, h	1180	930	380			
	,, o	1280	440	190	42	(13)	(12)‡
Brain	Anti-l	160	(65)	(26)			
	,, k	90	70	(25)			
	,, li	100	(60)	(9)			
	,, b	140	90	(25)			
	,, h	50‡	60	(5)			
	,, o	70	90	(12)			
Heart	Anti-l	1120	570	110			
	,, k	970	330	130			
	,, li	940	140	(27)			
	,, b	1170	280	150			
	,, h	440	310	140			
	,, o	400	190	(32)			
Spleen	Anti-l	3850	4850	1080			
	,, k	4390	2420	1170			
	,, li	1930	380	550			
	,, b	3110	1380	340			
	,, h	1990	1750	970			
	,, o	1020	460	780			
Blood	Anti-l	6700	710	150	(28)	(22)	(2)
	,, k	19700	3000	210			(15)
	,, li	8150	880	110	(28)	(9)	(0)
	,, b	8650	370	140			
	,, h	6980	480	150			
	,, o	14300	6180	120	(34)	(6)	64

* Count for wet, perfused tissues corrected to day of perfusion. Counts are averages of duplicate samples with a four-minute count and a background of about 20 cpm. Counts based upon original counts of less than 20 cpm, or less, above background are not accurate and are indicated by enclosure in parentheses.

† l, lung; k, kidney; li, liver; b, brain; h, heart; o, ovalbumin.

‡ Single sample.

In the case of the liver, previous work(5) showed that anti-mouse-kidney serum had a very low, but definite, localization with a long duration in the liver of mice. In this experiment with anti-rat-kidney serum there similarly was an indication of some localization in liver with a long duration. With anti-lung serum also, there seemed to be definite localization in the liver with a long duration. When compared with the antiovalbumin control, there was a suggestion of liver localization for anti-brain and anti-heart sera. On the other hand, the tissue-homologous serum, the anti-liver serum, showed essentially the least localization in liver. This may have been due to the fact that the rabbits producing the antiserum had received fewer injections of antigen over a shorter time, although actually good precipitins against liver tissue were present in the serum. In the case of the brain and heart there was no significantly greater localization from any of the sera, including the tissue-homologous serum, than from the antiovalbumin control.

The amounts of localization in the spleen were very variable, although there seems to have been somewhat greater localization initially from the anti-lung, -kidney, -brain, and -heart sera than from the anti-liver serum and the antiovalbumin serum control.

The amount of radioactivity in the blood indicated the relative amounts of radioiodinated protein present in the blood on the day of analysis, and was a function of the amount of radioactivity injected and the rate of removal of the radioactivity from the bloodstream. During the first few days after injection, the radioactivity in the blood may be taken as an indication of the amount of radioactivity actually injected. Therefore, it appears that the animals receiving anti-kidney serum and antiovalbumin serum may have received somewhat higher quantities of radioactivity than the others. Hence, the relative initial localization in the various tissues from these sera was somewhat less, on a comparative basis, than the counts would indicate. The preceding discussion of the results would not be altered by any such correction.

The failure to demonstrate tissue-specific localizing antibodies in the anti-liver, -brain, and -heart sera is not conclusive. This is emphasized by the fact that we know that there are substances in liver which bind antibodies prepared against kidney and lung, indicating that there must be common or at least cross-reacting antigens in these tissues. However, the antiserum prepared against liver not only did not react with the kidney or lung where cross-reacting antigens are known to be present, but also did not localize in liver as anti-kidney or anti-lung sera did.

These data show that the anti-lung serum localized in lung. The anti-lung serum also contained antibodies which cross-reacted with substances in the liver and kidney. Although the localization in the kidney from anti-lung serum was quite similar in extent to the localization from anti-kidney serum, the localization in lung was quite different for the two sera. Thus, there must be substances in the lung which are different from those in the kidney and which are responsible for the production and localization of anti-lung antibodies. It follows from the foregoing data that kidney tissue is not unique in being able to accumulate antibodies from tissue-homologous antiserum.

Summary. By labelling anti-rat-lung serum with radioiodine and injecting it into rats, it was found that the anti-rat-lung serum contained antibody which localized specifically in the lung. It also contained antibody which cross-reacted with and localized in the kidney. Anti-rat-brain, -liver, and -heart sera were also investigated and showed no specific localization, but these experiments were considered inconclusive.

The data presented show that kidney tissue is not unique in being able to localize antibody from tissue homologous antiserum.

We wish to thank Miss Beila Sherman, Miss Rhoda Michaels, and Mr. Richard Clarke for assistance.

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Protective Effect of Riboflavin on the Toxicity of 2-Acetylaminofluorene in Rats.* (17607)

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The carcinogen, 2-acetylaminofluorene, has been shown by Griffin *et al.*(1) to produce a reduction in liver protein and riboflavin in the rat. The depleting effects of this drug on the body stores of proteins and riboflavin in dogs have also been demonstrated.(2,3) Other carcinogens have been shown to produce riboflavin deficiencies in rats, the vitamin protecting the animal from the toxic effects of the chemical agent possibly by entering into its detoxication.(4,5) The following experiments were done to determine the protecting effects of dietary protein and riboflavin on the toxicity of 2-acetylaminofluorene in the rat.

Methods. Various amounts of riboflavin and 2-acetylaminofluorene were added to the following diet for rats, the values being expressed as percentages: casein 18, sucrose 13.4, glucose 20.2, dextrin 18.2, lard 24.2, Wesson's salt mixture 1.7,(6) agar 3.3, and cod liver oil 1. These constituents were made into a gel with water equal to 1.7 times the dry weight. The vitamin supplements as mg/kg of wet weight of diet were choline chloride, 1000; inositol, 100; thiamine, 2; ascorbic acid, 4; pyridoxine HCl, 1.6; 40 each of Ca pantothenate, niacin, p-aminobenzoic acid and α-tocopherol and 0.2 mg each 2-methyl,1,4-naphthoquinone, biotin and folic acid. The protein-free diet was made by replacing the casein with an equivalent number of calories in the form of glucose. The diet was pair fed to adult male Long Evans rats (300 g), each animal receiving 30 g per day. The rats were divided into 6 goups of 6 animals each (Table I). After 8 weeks of feeding, the rats were observed for skin lesions and the livers were analyzed for riboflavin.(7)

Results. The results of feeding different amounts of riboflavin to rats with or without 2-acetylaminofluorene in the diet are recorded in Table I. The data in Table I demonstrate that rats receiving 3 γ of riboflavin per day in the synthetic diet containing 18% casein did not show any evidence of a deficiency during the feeding period of 8 weeks. The riboflavin content of the livers averaged 16 γ/g of tissue. When 0.03% 2-acetylaminofluorene was added to the diet, skin lesions developed and the riboflavin content of the liver decreased to 9.5 γ/g of tissue. Thirty γ of the vitamin per day prevented the appearance of symptoms of deficiency in rats fed the carcinogen, the liver riboflavin being the same as the controls. In the absence of proteins in the diet, however, even 50 γ of riboflavin per day did not protect the rats from the toxic effects of 2-acetylaminofluorene. It is possible that the sparing action of protein may be in part through an effect of the protein on riboflavin. Reisen, Schweigert and Elvehjem,(8) for example, have demonstrated that an increase in the amount of protein ingested increased the riboflavin retention in the liver of the rat.

Summary. The carcinogen, 2-acetylaminofluorene, produced a riboflavin deficiency in rats, reducing the liver riboflavin below control values. Increasing the riboflavin content of the diet prevented the appearance of the de-

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TABLE I.
Skin Lesions and Riboflavin Content of the Liver Associated with Various Amounts of Riboflavin (R) and 2-acetylaminofluorene (AAF) in the Diet.

Group	R, γ/30 g diet	AAF %	Casein, %	Skin lesions	Liver riboflavin, γ/g tissue
1	3	0	18	—	16.0 ± 1*
2	3	.03	18	++++	9.5 ± 1
3	0	0	18	++	12.7 ± 0.9
4	0	.03	18	+++	9.3 ± 2
5	30	.03	18	—	16.0 ± 1.5
6	50	.03	0	+	9.9 ± 1

* Standard error.

ficiency, keeping the liver riboflavin at normal levels. In the absence of protein in the diet, however, the increased riboflavin did not prevent the development of the deficiency.

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Toxicity of Eugenol: Determination of LD50 on Rats.[*] (17608)

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As a result of the demonstration that eugenol (4-allyl-2-methoxyphenol) is a very effective mucigogue and desquamatory agent in the stomach,(1) this substance has been used for the study of mucus secretion and gastric cytology in man, without gastric resection or gastroenterostomy.(2) Our technic involves administration of an aqueous emulsion of the eugenol by stomach tube, and its reaspiration after about 15 minutes. Prior to such clinical application, however, it was deemed necessary to estimate the limits of safe dosage for this substance. Such a study, conducted on dogs without any gastric operation,(3) revealed a maximum safe dosage of about 0.2 g/kg body weight, when an aqueous emulsion was administered in this same manner without reaspiration. A dose of 0.25 g/kg caused no fatalities, but induced vomiting in 2 of 7 experiments. There was no gross or microscopic evidence of any cumulative effect after repeated administration of this dose—10 times in the course of 3 weeks. At dosage levels around 0.5 g/kg, reactions to the irritant, including vomiting and ataxia, were manifest and in 2 of 6 experiments, the dog died within 24 hours. Hence, a more precise determination of the toxicity, carried out on a large number of animals, was necessary. Such a study on rats is presented herein.

Experimental Procedure. About 200 albino rats, each weighing approximately 200 g, were used in this investigation. The animals, kept in individual wire cages, were fasted for 24 hours before the eugenol was administered and for about 4 hours thereafter. A total of 91 animals were included in the final dosage-mortality analysis. The individual dosage groups were so constituted that each contained equal numbers of males and females. Eugenol (Merck, U.S.P.) was administered undiluted in a single dose, in a manner essentially the same as that reported by Shay(4)

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for intra-gastric instillation in rats. If any resistance to passage of the catheter was encountered, the tube was removed immediately and passed a second time. Only 2 of some 200 rats were lost as a result of the intubation, and these 2 deaths occurred within 10 seconds after administration of the material. Since such accidental deaths were easily recognized, they were omitted from the results reported. All animals were observed closely throughout the day of administration of the eugenol, and were checked frequently for at least 10 days thereafter. All but 2 of the deaths occurred during the first 2 days. Those rats in which postmortem changes were considerable, because of death during the night, were discarded; all others were autopsied as soon after death as possible, and tissues from representative animals were taken for microscopic examination. Eugenol was administered in dosages based on body weight. In order to approximate the dosage range suitable for a dosage-mortality study, preliminary runs were made with several groups of 2 rats each. Based on these observations, more extensive runs were made in the range 1.5-2.2 ml/kg (1.6-2.3 g/kg) using 12 animals in each group. Whenever any doubt arose regarding the completeness of administration of the eugenol, the animal was immediately discarded from the group.

Results. Toxic manifestations. The first observable effect after the administration of eugenol was a weakness of the hind legs, which was seen in 100% of the animals. As a rule, this appeared in about 5 minutes, and by 15 minutes, there was complete paralysis of the lower extremities. Simultaneous with, or just after the onset of this hind leg effect, the lower jaw relaxed and remained in an open position for a variable period of time. This was observed in 70 (86%) of the animals. Sixty-four (70%) finally became prostrate and were unable to move, though eye reflexes were still present; 54 of these prostrate animals (60% of the total) went on to complete coma. In no case was an animal observed in which the front legs were affected before prostration or coma set in. Only 5 animals survived the coma; of the remaining 49, at least 10 were observed to arouse one or more times before death. During coma, breathing was irregular, changing from slow, deep, gasping excursions of the chest, to rapid, weak, abdominal respiration. The extremities became cyanotic and cold. Cessation of respiration usually occurred before heart failure. The time of death varied from 1 to 48 hours, 41% of all the deaths occurred within the first 6 hours, 33% in the next 18 hours, and 22% during the second day after administration of the eugenol. One animal died on the third day and one on the sixth. All survivors, whether they became comatose or not, were lethargic and manifested some degree of narcosis—some for as long as 3 days. On the second or third day, the animals usually sat hunched in a corner of the cage. After being prodded, they walked with hunched back and exaggerated high-step of the hind legs. Fifty per cent developed hematuria or urinary incontinence by the third day. By the fifth day, the survivors had all essentially recovered.

Pathology. In 38 (95%) of the 40 animals which were autopsied, eugenol odor was detected in either the stomach, the duodenum, or both. The blood vessels in the peritoneum and mesentery were engorged, as were those in the diaphragm, ribs, and genital organs. The peritoneal wall was glistening and diffusely inflamed, varying from pink to bright red, and fluid was present in the peritoneal cavity. There was diffuse congestion of the kidneys, and the medulla was often pink and poorly delineated. The liver also was usually congested and darker than normal. The glandular stomach contained mucus, clotted blood, and occasionally some areas of mucosal erosion; the fore-stomach appeared normal. Blood clots were often found in the lumen of the small intestine. The lungs were usually mottled and redder than normal. In many animals opened promptly after death, the auricles were still beating; in all instances, the ventricles were engorged and the vessels were injected. The general impression was that of a diffuse inflammation with congestion in the viscera.

The microscopic studies yielded evidence of pathologic change, especially following the use

TABLE I.
Dosage-Mortality Data for Eugenol.

Group No.	Eugenol dose, ml/kg	No. of rats per group	No. of deaths following administration (by days)					
			1*	2	3	4-10	Total	%
T-13	1.5	12	2 (2)	2	—	—	4	33
20	1.6	12	6 (3)	1	—	—	7	58
14	1.75	11	0 (0)	2	—	1†	3	27
16	1.75	11	1 (0)	1	1	—	3	27
17	1.9	12	3 (1)	1	—	—	4	33
15	2.0	11	7 (5)	1	—	—	8	73
22	2.1	11	8 (6)	1	—	—	9	82
21	2.2	11	9 (3)	2	—	—	11	100
	Total	91	36 (20)	11	1	1	49	
			74%	22%	2%	2%	54%‡	

* Values in parentheses give number of deaths in first 6 hrs.

† This death occurred on the sixth day.

‡ This represents the proportion of all deaths among all the animals treated, whereas the other percentages on this line give the proportions of all the deaths which occurred in the groups indicated.

of the highest dosages (2.1 and 2.2 ml/kg). Damage appeared to be greatest in the lungs, liver, kidney, stomach and duodenum, with relatively minor or no changes elsewhere. The lungs were emphysematous, with distended bronchi, and intense congestion was evident in the larger blood vessels as well as in the alveolar septa. In the liver, extensive congestion of the central veins and sinusoids was observed. At the higher dosages, the kidneys showed evidence of mild tubular dilatation, with the tubular epithelium swollen and distended with blood. The effects of eugenol on the nervous system in general were not studied histologically, but the possibility of their occurrence must be entertained in view of the gross observation of narcosis and malfunction of limbs and jaw following administration of the irritant. The wall of the forestomach was normal, showing no apparent reaction to the eugenol. The glandular stomach mucosa sometimes showed evidence of subacute inflammatory reactions with extensive leucocytic infiltration. In general, the columnar cells were distended with mucus, and desquamated masses of surface epithelium were noted in the lumen of the viscus. In the mucosa of animals given high dosages, areas of desquamation were distinctly visible. Parietal cells were well preserved, but the peptic cells were degenerate in appearance. Duodenitis was sometimes seen. There was evidence of excessive mucus production not only in the stomach, but also in the duodenum and other areas of the small intestine.

LD50. The mortality data are summarized in Table I. Using the statistical technique described by Bliss(5,6) the LD50 for eugenol in albino rats under the conditions herein described was found to be 1.8 ml/kg (1.93 g).

Antagonistic agents. Several stimulants were tested for possible therapeutic action in animals poisoned with eugenol: Picrotoxin (1 mg/kg), coramine (5 mg/kg), strychnine (0.75 mg/kg), caffeine (10 mg/kg), and metrazol (7.5 mg/kg). Each of these was tested on a group of about 10 rats that had received eugenol in single doses of 2.0 ml/kg. The drugs were injected intraperitoneally when the animals were already in coma or, if no coma occurred, when they were in deep narcosis. In some animals, repeated injections of the same dosage were made. No statistically significant reduction in mortality was observed in any of these experiments.

Summary. The LD50 for pure eugenol, administered to rats by stomach tube, and without subsequent reaspiration, was estimated to be 1.8 ml (1.93 g/kg). Any use of this datum as a guide to safe dosage in man,

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however, may take cognizance of the fact that the conditions of the rodent studies are considerably more severe than any which might be encountered in clinical studies. In the first place, the rat experiments employed eugenol *per se*, whereas work with dog and man is done with aqueous emulsions of the irritant. Furthermore, the rat retains all of the eugenol administered, because of its inability to vomit, whereas dog and man can reject part of the irritant in this manner if the dose is excessive, and this affords an additional factor of safety.

The salient features of the toxic manifestations of eugenol in the rat were as follows: Paralysis occurred initially in the hind legs and the lower jaw. The forelimbs were unaffected unless general prostration or coma ensued. In those animals in which the acute symptoms subsided, the animals remained lethargic, showed signs of urinary incontinence and frequently hematuria, and gave evidence of impaired function of the hind legs for several days. Gross and microscopic observations of the tissues suggested that profound changes in fluid distribution had occurred in response to acute irritation of the gastrointestinal tract. The net impression of the effect of the eugenol, from the microscopic evidence, was essentially that of circulatory collapse with resultant congestion.

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